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# **An Exploration of the Dynamics of Selection for Resistance to Herbicides**

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School of Life Sciences, University of Warwick

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## **Declaration**

This thesis is submitted to the University of Warwick for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree. All work presented has been undertaken by myself.

## Summary

Herbicide resistance in weeds is a major threat to agricultural productivity, but the incidence of herbicide resistance in agricultural systems continues to increase. It is increasingly recognised that sustainable weed management can only be achieved by considering the ecological and evolutionary drivers of herbicide resistance evolution. Within this context, it has been suggested that selection within pre-existing variation in herbicide susceptibility, underpinned by additive genetic variation, may result in rapid evolution of herbicide resistance. In this thesis, these principles were tested in the major agricultural weed *Alopecurus myosuroides* (black-grass). Dose-response experiments demonstrated pre-existing phenotypic variation in response to two commercial herbicides with alternative modes of action (the ACCase inhibitor herbicide fenoxaprop-P-ethyl and the ALS inhibitors mesosulfuron-methyl and iodosulfuron-methyl-sodium) in *A. myosuroides* with no history of exposure to herbicide. Selection within this pre-existing variation was then tested using low doses of the herbicide fenoxaprop-P-ethyl, demonstrating rapid increases in resistance following a single generation of selection. Recurrent selection showed further, but non-significant, increases in resistance. Competition experiments were then performed to compare fitness in the absence of herbicide between the original susceptible population, and a population following two generations of selection for fenoxaprop-P-ethyl resistance. Results suggested that both resistant and susceptible *A. myosuroides* populations were of similar fitness and competitiveness: seed output was highly variable, and both resistant and susceptible populations were highly competitive against wheat. Finally, the possibility of exploiting dose-dependent selection to limit resistance evolution was tested in a long-term dose rotation experiment using the unicellular alga *Chlamydomonas reinhardtii*. The results showed that rotating between a very high herbicide dose and no herbicide application could limit resistance evolution, but overall population control was poor. There were indications that rotation between intermediate higher and lower herbicide doses could successfully control population sizes and limit resistance evolution, but these effects were only present in the earlier stages of the experiment

# **1. Introduction**

## **1.1 Weed control in agriculture**

### **1.1.1 History of weed control in agriculture**

The transition to agriculture from previously foraging communities began approximately 11,000 years ago, and caused a demographic shift that has ultimately led to the current global human population of 7 billion (Bocquet-Appel, 2011). Cultivation inevitably supports other, undesired plants resulting in the proliferation and evolution of agricultural weeds (Perkins, 2002). Across much of the history of agriculture yields increased slowly, until scientific advances in the 19<sup>th</sup> century brought rapid gains (Evans, 1980). Weed control was, however, not initially a major research focus, and it is noted in the obituary of 19<sup>th</sup> century agriculturalist Sir John Bennet Lawes that while he experimented with chemical additions as fertiliser, weed control was performed manually (The Times, 1900).

In the 1940s the compound 2,4-dichlorophenoxyacetic acid, or 2,4-D, marked a major transition as the first synthetic herbicide to be commercially released (Quastel, 1950), starting an era of huge reliance on chemical controls after 1945 (Smith and Kennedy, 2002). This significantly changed the nature of farming, greatly reducing human labour and replacing it with chemical energy in pest management (Zimdahl, 2007). There are now over 300 chemicals used for herbicidal weed control (Heap, 2014a), classified into 16 different modes of action (Herbicide Resistance Action Committee, 2010), but no new major modes of herbicide action have been marketed for over 20 years (Duke, 2012).

### **1.1.2 Contemporary importance of agricultural pest control**

The global population is predicted to reach 9.6 billion by 2050 (Raftery et al., 2012; United Nations Department of Economic and Social Affairs, Population Division, 2013). Food production must increase to meet this growth, coming either from expansion of farmed areas or intensification and enhanced technology in lower-yielding countries (Tilman et al., 2011). There is however limited land suitable for arable farming, and agriculture must be considered as a component of wider land-use needs (Garnett et al., 2013). Current predictions suggest that 90% of the increased

food production is expected to come from yield gains, rather than expansion of farmed areas (FAO, 2009). It has even been suggested that we are at the point of 'peak farming', and over the next few decades the total farmed area could decrease (Ausubel et al., 2013). Our ability to achieve high yields is constantly compromised by pests, which can cause potential losses of up to 80% (Oerke, 2006), necessitating control.

Agricultural pests can be split into three major groups of animal pests, pathogens and weeds. These can cause losses through quantitative reductions in yields, or a decline in quality which reduces financial value – for example aesthetic attributes such as superficial damage or contamination with pests at harvesting. Of these three categories, weeds have been estimated to cause the greatest potential yield losses, equal to pathogens and animal pests combined (Oerke, 2006). The importance of weed control is further compounded as it has a much greater efficacy (at 74%) than non-virus pathogens (32%) or animal pest controls (39%) (Oerke, 2006).

### **1.1.3 Methods of pest control in agriculture**

The development of life sciences in the nineteenth century moved pest control to a scientific, technological approach. This era saw the emergence of synthetic chemical pesticides, such as the highly toxic arsenic based compound Paris Green as an insecticide against Colorado potato beetles in the 1860s, and Bordeaux mixture - copper sulphate and calcium hydroxide - as a fungicide against downy mildew in the 1880s (Perkins, 2002). In the 20<sup>th</sup> century the use of synthetic pesticides increased dramatically in scale and diversity, beginning with commercial use of the insecticide dichlorodiphenyltrichloroethane, DDT, in the 1940s. The extremely broad use and commercial success of DDT launched the synthetic pesticide industry, with many companies investing in the development of further insecticides, fungicides and herbicides in the following decades (Perkins, 2002).

Weed control remained reliant on mechanical or cultural methods until the 1940s and the development of the herbicide 2,4-D (Quastel, 1950). Problems soon developed due to an overreliance on chemical controls and initial ignorance as to their disadvantages. Secondary pest problems could arise as the initial pest control intervention killed predators, and cases of pesticide resistance soon arose (Smith and Kennedy, 2002). The publication of *Silent Spring* (Carson, 1962) brought issues

concerning pesticides to a wider audience, and showed the extent to which negative externalities could spread (Peshin et al., 2009). These environmental impacts and the risk of resistance evolution have led to a call for a more measured use of pesticides, and in the case of weed control, integration of non-chemical control methods (Harker et al., 2012). Non-chemical control methods are broadly split into cultural practices, which modify the cropping environment to reduce the extent and impact of weeds; and physical methods of removing weeds (Melander et al., 2005). Physical, or mechanical, controls are widespread and continue to be developed (Mohler, 2002; Vincent et al., 2001), and the efficacy of many cultural control methods has been demonstrated (Harker and O'Donovan, 2013). However, these cultural and mechanical controls generally only reach the high levels achieved by chemical control when they are applied as a combination of measures, each of which is typically much more labour-intensive (Bastiaans et al., 2008). Further potential avenues for non-chemical weed controls are currently being developed, including, for example, robotics and RNA-interference (Shaner and Beckie, 2014), and may well change the nature of weed control in the future.

#### **1.1.4 Chemical controls of pest plants**

There are a wide range of chemicals used as herbicides. Herbicides act by inhibiting the activity of an enzyme or enzymes required for normal growth and survival. Herbicides are typically defined by their target enzyme or process (table 1.1). For each herbicide mode of action multiple chemical families can exist, and within each chemical family there can be several specific active ingredients. Active ingredients of the same family can differ in their efficacy and risk of resistance evolution. The range of active ingredients available for use in individual crops and regions depends on current need and commercial status, and must be approved by a regional legislator. In the EU herbicide registrations are covered by regulation No. 1107/2009 (European Parliament, 2009). Assessments of active ingredients are made at EU level by the European Food Safety Authority. Member states then evaluate and authorise commercial products to be used at a national level. Usage will be restricted to a range of doses and application sites and/or methods.

Herbicides may be banned or restricted if their use is considered too damaging for the environment or poses too great a risk to human health. The environmental and

societal damage caused by pesticide use in the United States has been estimated at over \$8 billion per year (Pimentel, 2005). Risks to human health are often difficult to quantify, with unclear evidence for long-term exposure risks (Morrison et al., 1992). Safety of exposure to herbicides as they are applied is considered as part of the approval process, and may be updated if new concerns arise (for humans or the environment). Herbicide residue levels on food are further regulated by No. 396/2005 (European Parliament, 2005) within the EU. The relative advantages and disadvantages of pesticide use are complex but there is little doubt that there are many benefits (Cooper and Dobson, 2007), and herbicides are essential to maintain the scale of food production as farming is practised at present.

Table 1.1 Herbicide modes of action as grouped by the Herbicide Resistance Action Committee

HRAC group	Mode of Action
A	Inhibition of acetyl CoA carboxylase (ACCase)
B	Inhibition of acetolactate synthase (ALS)
C1	Inhibition of photosystem II
D	Photosystem I electron diversion
E	Inhibition of protoporphyrinogen oxidase
F1	Bleaching through inhibition of carotenoid biosynthesis at the phytoene desaturase step (PDS)
F2	Bleaching through inhibition of 4-hydroxyphenol-pyruvate-dioxygenase (4-HPPD)
F3	Bleaching through inhibition of carotenoid biosynthesis
G	Inhibition of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase
H	Inhibition of glutamine synthetase
I	Inhibition of dihydropteroate synthase
K1	Microtubule assembly inhibition
K2	Inhibition of mitosis / microtubule organisation
K3	Inhibition of very long chain fatty acids (VLCFAs)
L	Inhibition of cellulose synthesis
M	Membrane disruption
N	Inhibition of lipid synthesis
O	Synthetic auxins
P	Inhibition of auxin transport
Z	Currently unknown modes of action

## **1.2 Herbicide resistance in agricultural weeds**

### **1.2.1 History of herbicide resistance**

Despite their great importance for controlling weeds, herbicides risk losing efficacy as weeds evolve resistance. Concerns over the potential for herbicide resistance were first raised as far back as 1953 (Abel, 1954). The first herbicide resistant weeds were reported in 1957 (Hilton, 1957). Since then reported cases of herbicide resistance have consistently been increasing, with reports logged by the International Survey of Herbicide-Resistant Weeds (Heap, 1997, 2014a). As of July 2014 there are 236 weed species in which resistance has been reported (Heap, 2014a). Cumulatively, herbicide resistant weeds have been estimated as causing losses of \$33 billion annually in the United States alone (Zimmer, 2013).

### **1.2.2 Target-site resistance**

Herbicide resistance mechanisms are split into two major categories of target-site and non-target-site resistance. Target-site resistance (TSR) involves the targeted enzyme becoming insensitive to the herbicide, either through a structural change that reduces herbicide binding or via increased production of the target enzyme (Powles and Yu, 2010). Herbicide binding is prevented or limited by changes in the 3D structure of target enzymes and/or by changes to the distribution of polar amino acids at the herbicide binding site (Délye et al., 2013a). These changes are usually caused by substitution of an amino acid resulting from single nucleotide substitutions. More complex changes, including codon deletions and double mutations in a single codon (Han et al., 2012), have also been observed (Délye et al., 2013a). Increased production of the target enzyme can occur through gene amplification (Gaines et al., 2010) or gene regulatory changes which increase transcription (Baerson et al., 2002).

### **1.2.3 Non-target-site resistance**

Non-target-site resistance (NTSR) covers a broad range of mechanisms. For a herbicide to act, it must first enter the plant, then reach its target location (by translocation for systemic herbicides) and accumulate in sufficient quantities to bind and cause damage (Délye et al., 2013a). This results in multiple means by which



NTSR can operate. Reduced leaf absorption has been demonstrated, and is thought to act through changes to cuticle thickness and/or chemistry, but the exact mechanisms are as yet unknown, and this form of NTSR has rarely been demonstrated (Vila-Aiub et al., 2012). Following penetration of the plant, altered translocation can provide resistance by reducing herbicide translocation in the xylem or phloem, sequestering herbicide to the vacuole, or excluding herbicide from leaf cells or chloroplasts (Délye, 2013). NTSR mechanisms may also metabolically degrade a herbicide, or inactivate it by conjugation of an additional chemical group (Powles and Yu, 2010). Where herbicides cause damage by releasing active oxygen species, oxidases and peroxidases may act to limit oxidative damage (Cummins et al., 2013; Délye, 2013).

Though many NTSR mechanisms are still being resolved (Gaines et al., 2014), it is thought that NTSR frequently evolves as a polygenic trait: for example, metabolic degradation is a multistep process requiring several enzymes (Délye, 2013). NTSR resistance in individual plants is frequently provided by more than one mechanism (Powles and Yu, 2010), and a single gene may be responsible for NTSR through multiple pathways (Cummins et al., 2013). Many of the genes currently identified as responsible for NTSR belong to large enzyme families, such as cytochrome P450s and glutathione-S-transferases, which may have roles in multiple NTSR mechanisms and/or their regulation (Yuan et al., 2007). The enzyme families involved in NTSR are often components of wider plant stress responses (Das et al., 2010). Some non-target-site herbicide resistance mechanisms can therefore be considered as components of larger stress responses, which in resistant individuals are enhanced either constitutively, in response to the herbicide, or both (Délye, 2013). As many NTSR mechanisms are generic responses to a range of xenobiotics, they can provide resistance to more than one herbicide, known as cross-resistance (Powles and Yu, 2010), and selection by one herbicide or herbicide mode of action may also confer resistance to other modes of action to which the population has never been exposed (Busi and Powles, 2013). The evolution of cross-resistance appears complex however, as segregation of NTSR plants has shown that individual loci do not all provide cross-resistance to alternative herbicides (Petit et al., 2010), and further details of NTSR mechanisms are still being resolved (Gaines et al., 2014).

### **1.2.4 Evolution and inheritance of target site resistance**

The initial frequency of resistance alleles in naïve plant populations depends on spontaneous mutation rates, and any pleiotropic fitness costs resistance alleles confer. Theoretical estimates have been made predicting the frequency of major resistance alleles in unselected populations to be of the order of  $1 \times 10^{-5}$  to  $1 \times 10^{-6}$  (Jasieniuk et al., 1996), and collections from naïve populations (Darmency and Gasquez, 1990) and pre-herbicide herbarium samples (Délye et al., 2013b) have suggested even greater frequencies. Surveys of field resistance have revealed diversity in TSR resistance genotypes within small geographic areas, implying that TSR resistance alleles occur at reasonably high frequencies in weed populations, with independent mutations arising separately, rather than via rare mutation events and subsequent dispersal of resistance alleles (Délye et al., 2013a).

These TSR mutations are typically in nuclear genes that are inherited as dominant or semi-dominant traits at field application rates (Diggle and Neve, 2001). Recessive inheritance has also been reported, though the mode of inheritance was dependent upon the herbicide dose received (Délye et al., 2004). Inheritance may also be cytoplasmic, if the resistance gene is located in the chloroplast, as in resistance to triazine herbicides (Délye et al., 2013a). The chloroplast genome is predominantly maternally inherited, although pollen can contain some chloroplasts, and pollen transmission of triazine resistance has been observed (Darmency and Gasquez, 1981).

### **1.2.5 Evolution and inheritance of non-target-site resistance**

Weed populations can be highly genetically diverse (Menchari et al., 2007), and this genetic diversity may be expected to give rise to variation in phenotypic traits, including herbicide sensitivity (Délye, 2013). A limited number of the alleles responsible for the complex, polygenic NTSR mechanisms as described in section 1.2.3 above may be present in individual plants (Délye, 2013). These alleles individually provide only a limited tolerance to herbicides, but will be rapidly selected for following herbicide application within the range of herbicide sensitivity they confer (Neve et al., 2014). In a sexually reproducing species, alleles will recombine, increasing resistance as co-ordinated NTSR mechanisms arise through the accumulation of these alleles. Under this model, non-target-site resistance would

therefore evolve and be inherited as a quantitative trait. The extent of additive genetic variation for NTSR, and hence the potential for quantitative responses to selection, remains unclear, but there is evidence of NTSR being under complex polygenic inheritance (Petit et al., 2010). Experiments using *Lolium rigidum* have demonstrated that susceptible populations exhibit survival at low herbicide rates, suggesting initial frequencies of resistance contributing alleles are high (Neve and Powles, 2005a), and selecting within these rates can very rapidly lead to resistance (Neve and Powles, 2005b).

### **1.2.6 Potential epigenetic resistance**

There is increasing consideration given to the possibility that some herbicide resistance mechanisms may be epigenetically inherited (Délye et al., 2013a; Neve et al., 2014; Powles and Yu, 2010). Under epigenetic inheritance changes to the structure of the genome which determine gene expression are inherited without changing the DNA sequence of the genes themselves. This may occur via DNA methylation, modification of chromatin structure, or the presence of non-coding RNAs (Goldberg et al., 2007). A plant responding to environmental stresses may activate a response via these processes, which are then epigenetically inherited and the response maintained in its progeny. In plants, epigenetic inheritance has been shown for responses to biotic stress (Slaughter et al., 2012), Systemic Acquired Resistance (Luna et al., 2012) and elevated insect resistance (Rasmann et al., 2012). Given the generic nature suspected of many non-target-site resistance mechanisms it would not be surprising if some of these mechanisms, or similar, could also contribute towards herbicide resistance. At present, however, no epigenetic inheritance of herbicide resistance has been demonstrated.

## **1.3 Ecological and evolutionary factors in selection for herbicide resistance**

### **1.3.1 Selection pressure**

As described above, herbicide resistance operates through a number of mechanisms that may already be present within a susceptible population, either a single major effect resistance allele or multiple minor effect alleles. Following herbicide exposure, any individuals with sufficient resistance can survive and pass on these

alleles to the following generation, selecting for herbicide resistance. Whether the mechanisms an individual plant has are sufficient to survive, and hence which mechanisms can be selected for, will depend on what dose is applied. Herbicide dose will determine the relative fitness of individual plants with different combinations of herbicide resistance alleles, and thus which will be selected (Neve et al., 2014).

Lower herbicide doses may be within the pre-existing phenotypic variation for herbicide sensitivity, resulting in rapid increases in resistance as described above. As countries legislate to reduce doses for environmental reasons, concerns have been raised over this potential evolutionary consequence (Gressel, 2009). In contrast, many target-site resistance single resistance genes have been shown to confer a very high level of resistance, and so any TSR mutations that arise will provide resistance largely irrespective of herbicide dose applied. If doses are sufficiently high that only major effect TSR alleles confer resistance, any mutations that do provide this high level resistance will rapidly spread through the population under a strong selective sweep (Délye et al., 2013a). This has been raised as a concern in herbicide resistant GM crops which encourage strong reliance on controlling weed populations with high herbicide doses (Owen and Zelaya, 2005). This therefore presents a difficult situation for weed management, where using either low or high herbicide doses is predicted to select for an alternative form of resistance (Gressel, 2002).

If using multiple herbicides, selection for resistance must be considered for each herbicide. The selection pressure for one herbicide may produce a correlated response for alternative herbicides, through broad NTSR mechanisms. Predictions are difficult however, as although plants may contain multiple NTSR alleles leading to resistance to one herbicide, only some of these contribute to resistance to others, and this subset may differ depending on the alternative herbicide (Petit et al., 2010). For target-site resistance selection pressures will generally work independently for different herbicide resistances, requiring independent TSR mutations unless they share the same mode of action. In some cases TSR selection pressures might also work antagonistically, where resistance to one herbicide results in increased sensitivity to others, termed negative cross-resistance, as recently shown for ALS inhibitor resistant *Kochia scoparia* being more susceptible to PPO and HPPD inhibitors (Beckie et al., 2012; Gressel, 2009).

### 1.3.2 Fitness costs

Herbicide resistance is clearly beneficial to fitness in allowing plants to survive herbicide exposure, but it may confer associated negative pleiotropic costs in the absence of the herbicide. These negative costs might be expected for a number of reasons: the mutations responsible for resistance could reduce normal enzyme function; increased resource allocation for resistance mechanisms may limit resources for growth or reproduction; or if an ecological interaction is compromised, such as disease resistance, then ecological costs of resistance will arise (Vila-Aiub et al., 2009a). The potential for negative cross-resistance to alternative herbicides (Beckie et al., 2012; Gressel, 2009) also represents a further resistance associated fitness cost, depending on the resistance mechanisms present and herbicide plants are exposed to. Pleiotropic effects across a weed's complete life cycle ultimately determine fitness. If being resistant confers a major disadvantage when not exposed to herbicide, the evolution and spread of resistance will be slowed, as fewer viable offspring may be produced, which may then also be at a competitive disadvantage (to other species, including the crop, or susceptible plants of the same species).

### 1.3.3 Mating system and gene flow

Flowering plants have exceptionally diverse breeding systems (Barrett, 2002), which will determine the inheritance and spread of resistance. In predominantly selfing weeds, e.g. *Conyza canadensis* the reduced heterozygosity will slow the spread of dominant major effect alleles in contrast to obligate outcrossers such as *Alopecurus myosuroides*. Conversely, this will mean recessive major effect alleles can spread more easily in selfing species (Diggle and Neve, 2001). For polygenic traits there is reduced opportunity for additive alleles to recombine in selfing species, but the degree to which this is important will depend on the number of genes involved and how they interact epistatically (Renton et al., 2011).

For outcrossing plants, alleles can be transferred between plants via pollen. Pollen fertilisation may occur across very long distances, and has been implicated in the spread of resistance across fields separated by several kilometres (Busi et al., 2008; Délye et al., 2010a). For both selfing and out-crossing species seeds also provide a means of dispersal, spreading short distances within a field or longer distances between fields via wind (Okada et al., 2013) or as a result of accidental

transportation with grains and/or farm equipment (Busi et al., 2011; Gaines et al., 2007). Once a population has a very high proportion of resistant individuals, resistance alleles are likely to spread and can be introduced to another field, although evidence suggests most populations have evolved resistance independently (Délye et al., 2013a). As well as these spatial introductions, the ability of seeds to remain viable within a seed bank for several years also gives an opportunity for alleles to be introduced from a temporally distinct source (Maxwell et al., 1990). The seed bank may provide a refuge for susceptible individuals and slow evolution by reintroducing non-resistant alleles to the population. Conversely, the seed bank may also provide a reservoir of resistance alleles when the population is no longer under selection by a given herbicide.

## **1.4 Management of herbicide resistance**

### **1.4.1 Multiple herbicides**

Management strategies can be employed to limit herbicide resistance if its evolution is understood. In general, strategies must diversify selection pressures in order to reduce the selection for any single form of herbicide resistance (Norsworthy et al., 2012). Using multiple herbicides with different modes of action is the most commonly advocated strategy for slowing or preventing the evolution of resistance (Délye et al., 2013a); where different herbicides can be applied either in rotation across alternate growing seasons or together in mixtures (Beckie and Reboud, 2009). In rotation strategies, there will only be selection for specific resistance mechanisms in the presence of that herbicide. Furthermore, resistance may confer pleiotropic fitness costs which result in selection against a given resistance mechanism in the presence of alternative herbicides. Models have suggested that cycling herbicide modes of action can limit resistance evolution, but only when pleiotropic fitness costs are incurred (Roux et al., 2008). A strong form of pleiotropic cost which can be exploited by herbicide rotation strategies is negative cross-resistance, as described above, where resistance to one herbicide mode of action results in hypersensitivity to others. Negative cross-resistance has been proposed as contributing to sustainable resistance management strategies (Pittendrigh and Gaffney, 2001), but can only be exploited where specific resistance mechanisms result in increased sensitivity to alternative herbicides. Negative cross-resistance has been implicated in the success

of some herbicide rotation strategies (Gressel and Segel, 1990), and has also been suggested for insecticide resistance management (Kolaczinski and Curtis, 2004). Applying herbicides together in mixtures has been suggested as potentially offering greater protection against resistance, as individual plants must evolve mechanisms to survive more than one herbicide (Beckie and Reboud, 2009).

Strategies involving the use of multiple herbicide modes of action may, however, also select for cross-resistance to multiple herbicides (Neve et al., 2014). Cross-resistance has been documented for many herbicide resistance mechanisms, and is an important concern for resistance management (Beckie and Tardif, 2012). Cross-resistance can confer resistance within populations to herbicide modes of action to which those populations have not previously been exposed (Busi and Powles, 2013). Promoting the evolution of generalist herbicide cross-resistance would therefore not only reduce the available options for weed control when resistance to one mode of action evolves, but also risks compromising novel herbicides, reducing the potential means of controlling weeds in the future. If there is a risk of cross-resistance across multiple modes of action, herbicides should be chosen which differ in their NTSR mechanisms, where known (Délye et al., 2013a).

#### **1.4.2 Variable dose strategies**

The principles outlined above for successful resistance management using herbicide rotations depend upon the individual herbicides selecting for independent resistance mechanisms (Neve et al., 2014). As described in section 1.3.1, different herbicide doses may result in selection for alternative resistance mechanisms, offering a similar opportunity to limit resistance evolution if varying doses are employed in a dose rotation strategy (Gardner et al., 1998). Further ecological dynamics may then add to the success of dose manipulation strategies: if, for example, susceptible plants germinate from the seed bank in low dose or non-herbicide periods in a rotation, these susceptible plants may outcompete resistant individuals, and/or dilute resistance allele frequency in the progeny (Gressel, 2009). Dose rotation strategies have been tested, and shown to be successful, in theoretical models (Gardner et al., 1998), but not yet demonstrated in experimental systems.

### **1.4.3 Non-chemical control strategies**

There is a diverse range of non-chemical control, which can be used to limit continuous selection for herbicide resistance (Harker and O'Donovan, 2013). Employing cultural controls and herbicides together in integrated systems can be very effective at controlling weeds, and is recommended to limit herbicide resistance evolution (Gill and Holmes, 1997; Moss and Clarke, 1994). Ensuring that weed densities remain low through cultural control when herbicides are losing efficacy will also limit reproduction of resistant plants. This will prevent large numbers of resistance alleles building up within the seed bank, and limit the likelihood of resistance spreading to neighbouring fields (Beckie, 2006; Busi et al., 2011). Despite these advantages, and legislation requiring integrated non-chemical controls in pest management (Lutman et al., 2013), there is a continued lack of integrated weed management research, and options for non-chemical control are frequently overlooked (Shaner and Beckie, 2014).

## **1.5 Implications in wider evolutionary biology**

### **1.5.1 Parallels in resistance management in other pests**

The evolution of pesticide resistance is not unique to plants. Insecticide resistance shares many of the issues concerning herbicide resistance, and limiting insecticide resistance is a similarly complex problem, requiring strategies to be developed which are backed up by evolutionary theory (Onstad, 2007). Insecticide resistance poses additional problems as it needs to be managed for the spread of disease vectors, including for humans, with different control techniques and aims (Kelly-Hope et al., 2008). While not currently underpinned by a similarly developed theoretical understanding, pesticide resistance in mammals too shows similar problems; for example warfarin resistance in rats appears to be possible through target-site (Lasseur et al., 2005) and non-target-site (Ishizuka et al., 2007) resistance mechanisms which could be considered analogous to herbicide resistance. Many parallels also exist in cancer treatment, as controlling selection pressures to limit the probabilities of individual cancer cells evolving resistance need to be considered similarly to individual plants in a large population, and multiple-drug resistance in



cancers is thought to act similarly to NTSR multiple herbicide resistance in plants (Cummins et al., 2013).

### **1.5.2 Antibiotic resistance**

There is increasing concern over the global increases in antibiotic resistance, with potentially devastating consequences for medicine (McAdam et al., 2012). Similar to selection pressures for herbicide resistance, issues concerning appropriate dose strategies have been highlighted in reference to the need for full courses of antibiotics to be completed. It has been demonstrated that at low, non-lethal, antibiotic concentrations resistance mutations are still selected, and in common with herbicide resistance, the previous focus on strongly selected, major effect mutations may miss important evolutionary steps (Hughes and Andersson, 2012). The range of drug concentrations microbes may be exposed to in different parts of the body will provide different selection pressures akin to alternative herbicide doses, and has been suggested as a means of rapid antimicrobial resistance evolution (Hermesen et al., 2012). Even extremely low antibiotic concentrations as might be found in the environment have been shown to select for resistance (Gullberg et al., 2011).

### **1.5.3 Evolution via major or minor effect genes**

Herbicide resistance evolution can also be used to inform very broad questions in evolutionary biology. As has been described above, selection for herbicide resistance can occur through standing genetic variation in minor effect NTSR mechanisms or major effect target site mutations. The relative occurrence of these modes of adaptation and their impacts are a key topic in evolutionary ecology (Barrett and Schluter, 2008). Another debate concerns the visibility of this pre-existing genetic variation, and whether it is only revealed when novel selection is imposed (McGuigan and Sgrò, 2009; Le Rouzic and Carlborg, 2008). NTSR to herbicides provides an excellent and pressing study system to explore this.

## **1.6 Herbicide resistance in *Alopecurus myosuroides* (Black-Grass)**

### **1.6.1 *Alopecurus myosuroides* as an agricultural pest**

*Alopecurus myosuroides* (black-grass) is the most important herbicide resistant weed in North-Western Europe (Moss et al., 2007). Black-grass is the greatest weed

problem for winter cereals such as wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), the two most widely grown crops in the UK (Department for Environment, Food & Rural Affairs (DEFRA), 2013), and is also a pest in their major break crop oilseed rape (*Brassica napus*) (Lutman et al., 2013). As well as causing substantial yield loss by competing with the crop (Lutman et al., 2013) black-grass is also prone to the same strain of the toxic fungus ergot (*Claviceps purpurea*). Ergot infected black-grass can contaminate the grain when harvested and spread the fungus to the crop itself during the growing season (HGCA, 2002; Mantle et al., 1977). Ergot fungus produces alkaloids with a range of dangerous physical and neurological effects on humans and animals (Eadie, 2003), which can lead to death, and as such contaminated grain is strictly prevented from being used commercially (Lorenz and Hosney, 1979).

### **1.6.2 Basic biology of *Alopecurus myosuroides***

*A. myosuroides* (black-grass) is an annual grass, normally growing to 20-80cm tall and producing dense, narrow panicles 2-12 cm in length, and can be a range of colours, from yellow to dark purple-green, giving the name. Spikelets are 4.5 – 7mm long with glumes connected before the base (around 1/3-1/2 of the way up the spikelet), distinguishing them from other members of the genus *Alopecurus* (Rose, 1989). *A. myosuroides* is a serious problem on arable farmland and as of 2011, 53% of arable farms, spread across most of Britain, used herbicides specifically to control *A. myosuroides* (Bayer Agri Services, 2012). *A. myosuroides* can also be found on other cultivated ground and wastelands, generally on heavier soils (Rose, 1989).

*A. myosuroides* can grow in densities up to almost 3500 plants per square metre, but rapidly reaches a density dependent self-thinning to produce a carrying capacity of approximately 1500 flower heads per square metre (Moss et al., 2010). Competition changes the growth form of black-grass, generally making plants grow taller with fewer tillers (Chauvel et al., 2005). Numbers of spikelets per panicle vary considerably and scale with head length, but can be up to almost 200 (Dalbiès-Dulout and Doré, 2001). Seed viability has also been shown to vary, reaching up to almost 90%, and it has been suggested that as an outcrossing species this scales with pollen availability (Chauvel et al., 2005). In UK fields *A. myosuroides* sheds its seeds rapidly after maturity, beginning in June and continuing into August (Swain et

al., 2006). Seeds show variable dormancy, correlated in particular with air temperatures (Swain et al., 2006). Seeds can remain dormant in the seedbank for at least 4 years (Moss, 1985) and potentially much longer.

### **1.6.3 Control of *Alopecurus myosuroides***

*A. myosuroides* has been predominantly controlled by herbicides (Lutman et al., 2013). Many herbicides have been used in the control of black-grass, including PSII inhibitors (chlorotoluron and isoproturon), ACCase inhibitors (fenoxaprop, clodinafop, propaquizafop, fluazifop-P-butyl and cycloxydim), ALS inhibitors (mesosulfuron and iodosulfuron mixtures), and tubulin formation inhibitors (propryzamide) (Lutman et al., 2013). Several pre-emergence herbicides are also important in *A. myosuroides* control, in particular: prosulfocarb, pendimethalin, flufenacet and diflufenican (Bailly et al., 2012). A range of cultural practises are also used, including spring cropping or delayed autumn drilling, more competitive crop cultivars, mouldboard ploughing and stubble clearance (Moss and Clarke, 1994). To both minimise the use of herbicides and limit resistance, integrated management strategies combining chemical and cultural controls are recommended to control *A. myosuroides* and are now included in EU legislation (Lutman et al., 2013; Moss et al., 2010).

### **1.6.4 Extent of herbicide resistance in *Alopecurus myosuroides***

*A. myosuroides* populations have already evolved resistance to all modes of action commonly used for its control except propryzamide and glyphosate (Lutman et al., 2013). A number of TSR mechanisms have been identified in black-grass, but it is increasingly widely acknowledged that NTSR is extremely important (Délye et al., 2011a). As well as providing resistance directly, including cross-resistance against multiple herbicides, NTSR could be implicated in the development of TSR by larger population sizes following herbicide application resulting in increased mutation supply (discussed further in chapter 3). Herbicide resistance in black-grass is extremely widespread across Europe, but the mode of resistance (including specific mechanisms within both TSR and NTSR) does not appear to be grouped by location (Délye et al., 2010b). Instead, resistance must have been able to evolve independently, with differences more likely driven by selection pressures and stochastic mutation events within individual fields (Délye et al., 2010b). The ease

and rapidity with which black-grass appears to evolve herbicide resistance makes it vital that weed control strategies incorporate evolutionary theory.

## **1.7 Models and model organisms in the study of herbicide resistance**

### **1.7.1 Models and predictions of herbicide resistance and its impacts**

Many of the questions we need to address concerning weeds involve large population sizes and selection across many years, making them extremely difficult to study experimentally. For example, though it is important to understand the dynamics of black-grass resistance, it is an annual plant which evolves resistance in field populations of tens of thousands of plants over several years, making replicable, controlled experiments impossible at this scale. If the processes driving weed population and evolutionary dynamics are well enough understood, we can instead use predictive models to successfully influence management practices (Freckleton and Stephens, 2009). Predictive models can also identify where our current knowledge is limited and direct research to fill in gaps in fundamental eco-evolutionary processes, in turn leading to more accurate models and more advantageous management strategies (Neve et al., 2009).

### **1.7.2 Model organisms**

Model organisms are widely used in biology to conveniently study processes thought to be shared among related species. The plant *Arabidopsis thaliana* is widely used as a botanical model species as it can be grown rapidly and takes up little space, and its genome has been fully sequenced with an extensive range of genetic resources (Mitchell-Olds, 2001). For example, *A. thaliana* has been used in herbicide resistance research to estimate the frequency of mutations conferring resistance (Jander et al., 2003) and interactions among multiple resistance genes (Roux et al., 2005).

The unicellular alga *Chlamydomonas reinhardtii* has also been used as a model organism for herbicide resistance, as although it is not as closely related to weed species of interest, it is still susceptible (and can evolve resistance) to many higher plant herbicides (Reboud et al., 2007). Due to the small size and rapid generation

time of microorganisms they are extremely useful for the experimental study of evolution (Elena and Lenski, 2003). *C. reinhardtii* has been used to study a number of aspects of the evolution of herbicide resistance, including fitness effects of evolving resistance (Vogwill et al., 2012), and the evolutionary consequences of herbicide mixtures (Lagator et al., 2013a) and cycles (Lagator et al., 2013b).

## **1.8 Major project objectives**

This project aims to explore some of the evolutionary and ecological processes that lead to herbicide resistance. In doing this the intention is both to develop applied principles in order to most successfully manage agricultural systems and advance this area of evolutionary ecology. The project does this first by examining pre-existing variation in responses to low levels of herbicide using the agricultural weed *Alopecurus myosuroides*. Low dose selection experiments then explore how selection within this variation can drive the early stages of resistance evolution, and how the herbicide dose applied influences these dynamics. The fitness consequences of having selected for herbicide resistance are then studied in competition experiments. Finally, using *Chlamydomonas reinhardtii* as a model organism, the longer term impacts of using different herbicide doses and cycling between them are tested.

## **2. Variation in response to ACCase- and ALS-inhibitor herbicides in susceptible *Alopecurus myosuroides* populations**

### **2.1 Introduction**

Historically, herbicide resistance research has focussed on the evolution of major effect resistance alleles (section 1.2.3), but it is increasingly recognised that a range of non-target-site resistance mechanisms are also extremely important (Délye et al., 2011a). Variation within these mechanisms may cause phenotypic resistance to be distributed among a population and inherited as a quantitative trait (Délye, 2013), with important implications for the evolution of herbicide resistance (Gressel, 2009).

In a naïve population without prior exposure to herbicides there may already be variation in susceptibility to herbicides resulting from pre-existing diversity in either mechanism described above (chapter 1.2.3 and 1.2.4). Herbicide application will then drive the evolution of herbicide resistance by selection from this pre-existing variation. If low-level, dose-specific resistance exists in the population as a quantitative trait some individuals will survive low herbicide doses. In an outcrossing species, the additive genetic variation responsible for this phenotypic variation can then recombine, providing resistance to higher doses in the following generation. Rapid evolution of resistance as a result of repeated selection at low doses has been demonstrated for *Lolium rigidum*, where initial frequencies of low-level resistance (resistance to relatively low herbicide doses) were high (Neve and Powles, 2005b). Alternatively, major effect alleles which contribute strong resistance will be selected for at any dose to which they confer survival, and will then spread through the population by a selective sweep. Which mechanism of herbicide resistance initially evolves will depend on what pre-existing diversity exists in the naïve population and the degree of resistance this provides to herbicide dose received.

The major effect alleles in herbicide resistance are predominantly mutations to the herbicide target enzyme that result in reduced herbicide binding, resulting in target-site resistance (TSR). Frequencies of these alleles within a susceptible population depend upon the mutation rates of the herbicide target genes, any fitness costs they impose in the absence of herbicide, and gene flow from other populations (chapter

1.2.3 and 1.3.3). Mutation rates in plants have been estimated at  $7 \times 10^{-9}$  substitutions per base-pair per generation for the model plant *Arabidopsis thaliana* (Ossowski et al., 2010), though this is yet to be confirmed in other species. Should they arise, the persistence of these major effect resistance alleles in a population before selection will be determined by genetic drift and whether they confer a fitness cost. Theoretical estimates have suggested the frequency of major resistance alleles in susceptible populations to be of the order of  $1 \times 10^{-5}$  to  $1 \times 10^{-12}$  (Jasieniuk et al., 1996) depending on herbicide mode of action. Target-site ALS (acetolactate synthase) inhibiting herbicide resistance mutations in unselected *L. rigidum* have been shown to be even more frequent than this, at  $1 \times 10^{-5}$  to  $1.2 \times 10^{-4}$  (Preston and Powles, 2002).

The minor effect resistance alleles that contribute to quantitative resistance traits are thought to confer non-target-site resistance (NTSR) mechanisms (chapter 1.2). Genome-wide mutation rates will govern the frequency with which enhanced NTSR mutations arise too, but as there are multiple genes that can contribute the likelihood of individual plants already having a minor gene mutation is greater. Mutation rates have been suggested of the order of  $10^{-2}$  per gamete per generation across all genes contributing to a quantitative trait (Lande, 1983). Variation within NTSR might also already be selected for prior to herbicide application as many of the genes involved belong to diverse stress-response pathways, and could be selected for enhanced ability to tolerate a range of abiotic stresses (Délye, 2013). As a result of these dynamics, resistance as a quantitative trait is expected to be driven through standing genetic variation readily found within weed populations, in contrast to TSR, the occurrence of which depends on the spontaneous mutation rate of major effect resistance alleles (Délye et al., 2013a).

Pre-existing variation in herbicide susceptibility among individuals may be observed as differential levels of survival across a range of herbicide doses. As this is determined by the herbicide environment, variation in phenotypic resistance cannot be directly measured in a standardised uniform environment, as might be done to measure variation in a morphological trait. Instead, the level of resistance in a population is commonly quantified via dose-response assays (further details in methods section below) which describe how plants respond to increasing herbicide doses. Dose-responses can, in principle, also be used to infer whether variation in

phenotypic resistance exists as a quantitative trait. Instead of measuring the value of the trait directly, for each herbicide dose applied the proportion of survivors is the proportion of the population for which their maximum survivable herbicide dose is at this dose or lower. If the underlying quantitative trait is normally distributed in the population, the dose-response obtained as described above will be the cumulative distribution function (CDF) of this normal distribution, as shown in figure 2.1. Differentiation of this CDF dose-response would then give the underlying trait distribution, and integrating the trait distribution would give the CDF. The CDF therefore has an inflexion point at the mean of the distribution, and the slope of the CDF reflects the variance about the mean of the trait distribution.

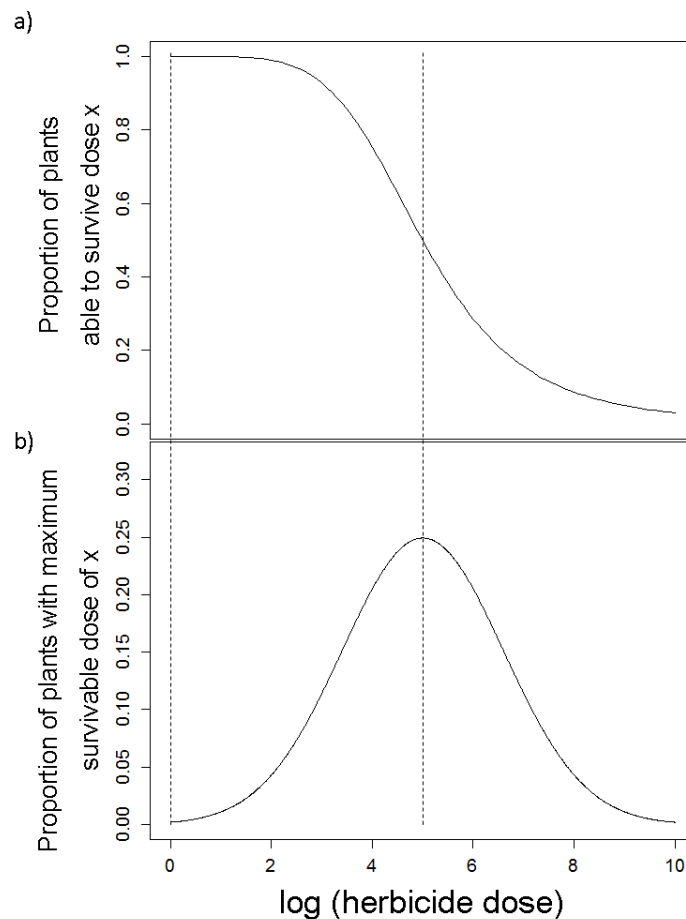


Figure 2.1 A model describing variation in maximum survivable herbicide dose for individual plants as a normally distributed trait (b), which corresponds to a cumulative distribution function (a) of the proportion of plants able to survive a given dose – the cumulative total of all plants whose maximum survivable dose is above the dose received. The LD<sub>50</sub>, the dose at which 50% of a population will be killed (or conversely, 50% of the population are able to survive), is shown to occur at the mean resistance phenotype, in this hypothetical population at a dose of 5 (arbitrary units).



In practice the phenotypic distribution will reflect not only the underlying quantitative trait (assumed in fig. 2.1 to be normally distributed), but also interact with environmental factors (Falconer et al., 1996). In the case of herbicide resistance the dose-response will include toxicological effects such as herbicide and adjuvant chemistries and interactions (Seefeldt et al., 1995). The dose-response of a population therefore reflects the underlying trait distribution as it responds to all toxicological effects. In herbicide resistance the log-logistic function (see methods below) is used as a standard model for dose-response relationships, including in this study, as it provides a robust fit for most experimental data and the parameters of the function are biologically informative and allow convenient comparisons. In practice, the log-logistic and normal distributions (and logistic distribution) are likely to be able to fit dose-response data similarly well within the range of doses and replication normally feasible in higher-plant herbicide studies.

In this section the conceptual model described above is used to explore the presence and extent of variation in response to herbicide application in susceptible *Alopecurus myosuroides* (black-grass). Multiple target-site resistance mutations have been identified in *A. myosuroides*, giving resistance to a range of herbicides, including both ACCase and ALS inhibiting herbicides, as used in this experiment. For ACCase inhibitor herbicides six separate amino acid substitutions have been shown to confer resistance in *A. myosuroides*: I1781L (Brown et al., 2002; Délye et al., 2002), I1781T (Kaundun et al., 2013a), W2027C, G2096A, D2078G (Délye et al., 2005), and I2041N (Délye et al., 2003). For ALS inhibitor resistance three amino acid substitutions have been identified: two replacing proline 197; P197H (Krysiak et al., 2011) and P197T; and W574L (Délye and Boucansaud, 2008; Marshall and Moss, 2008; Marshall et al., 2013). A large range of additional substitutions, at the same and alternative amino acid residues, have been shown to confer resistance in other species for both ACCase (Kaundun, 2014) and ALS (Yu and Powles, 2014) inhibitor herbicides.

The mechanistic basis for TSR is well understood at a molecular level, but the importance of NTSR is now increasingly being recognised (Délye et al., 2011a). The genetic basis for NTSR in *A. myosuroides* remains relatively little known, but recently a role was suggested for a single glutathione transferase gene *AmGSTF1* in providing NTSR by scavenging oxygen radicals (Cummins et al., 2013), and

cytochrome P450 herbicide detoxification has previously been reported (Letouzé and Gasquez, 2003). Other cases of NTSR have been shown to be under multigenic control, though as yet mechanisms have not been confirmed (Petit et al., 2010). NTSR degradation mechanisms have also been demonstrated in the majority of *A. myosuroides* plants also containing TSR (C. Knight, unpublished work). Specific TSR mutations differ in the level of resistance they provide (Kaundun, 2014), but it is thought that many TSR mutations observed in *A. myosuroides*, provide a very high level of resistance, which would make additional NTSR mechanisms redundant. The existence of both mechanisms in the same plant may suggest that lower levels of NTSR were initially present before the strong resistance of TSR later evolved.

This study explores the responses of two susceptible *A. myosuroides* populations to three commonly used agricultural herbicides. Results are interpreted to infer whether additive genetic variation is present in these populations at sufficient frequencies to survive the doses applied. The three herbicides used are commercial formulations covering two different modes of action: the acetyl-CoA carboxylase (ACCase) inhibitors, fenoxaprop-P-ethyl and cycloxydim, and a mixture of the acetolactate synthase (ALS) inhibitors mesosulfuron-methyl and iodosulfuron-methyl-sodium.

## **2.2 Materials and methods**

### **2.2.1 Plant materials**

Two susceptible *A. myosuroides* populations without previous exposure to herbicide were obtained from Herbiseed ([www.herbiseed.com](http://www.herbiseed.com)). One population was collected from an organic farm in Berkshire and is subsequently denoted ‘Berks’. The other population, denoted ‘Oxon’, was originally collected from an organic farm in Oxfordshire in 2004 and maintained as an isolated breeding population with no exposure to herbicides.

### **2.2.2 Herbicide dose-response assays**

In November 2010 seeds were sown in 15cm diameter pots filled with ‘J Arthur Bowers Top-Soil’. Seeds were sown at 1.5cm depth at 21 equally spaced locations within pots. Two seeds were sown at each location and following sowing seeds were covered with soil. Pots were maintained in a polythene tunnel at approximately 1°C

above ambient air temperature. Pots were watered daily. Germination and growth rates were initially slow due to cold temperatures, so in early December all plants were moved to a glasshouse heated to 10°C in the day and 5°C at night. In both locations pots were arranged in the same 3 replicate blocks with complete randomisation within blocks.

In January 2011 plants were thinned out so that each double sown location contained only a single plant at the 1.5-3 leaf stage to be treated with herbicide, resulting in an average of 15 plants per pot. All three herbicides were applied using a Berthoud Velmorel 200 pro 16 litre pump-operated knapsack sprayer operating at a pressure of 3 bar. A Hypro Flat Fan VP 110° nozzle was used (BCPC nozzle code F110/1.2/3) which gives a spray of fine droplets and output at a flow-rate of 1 litre per minute. During spraying pumping was maintained at a regular rate to maintain full pressure. Herbicides were applied in a total volume of 165 litres per hectare maintained by setting a constant walking speed with markings on the ground and setting pace with an electronic metronome.

All three herbicides were applied at 8 different doses equivalent to 0.05, 0.1, 0.2, 0.3, 0.5, 0.75, 1 and 2 times the UK label recommended rates for *A. myosuroides* control. For fenoxaprop-P-ethyl these doses corresponded to (normal label rate in bold) 2.74, 5.48, 10.96, 16.43, 27.39, 41.09, **54.78** and 109.56 g active ingredient ha<sup>-1</sup>. No adjuvant was recommended for use with the fenoxaprop-P-ethyl formulation. For cycloxydim the spray rates corresponded to 10, 20, 40, 60, 100, 150, **200** and 400 g active ingredient ha<sup>-1</sup>. For the mesosulfuron-methyl and iodosulfuron-methyl-sodium mixture the rates corresponded to 20, 40, 80, 120, 200, 300, **400** and 800 g product per ha<sup>-1</sup>, which contained 30g/kg mesosulfuron-methyl and 6g/kg iodosulfuron-methyl-sodium. For every application of cycloxydim and the sulfonylurea mixture a 6.7% by weight 3,6-dioxaeicosylsulphate sodium salt + 20.2% by weight 3,6-dioxoactadecylsulphate sodium salt adjuvant was included at 1 litre ha<sup>-1</sup>. Due to a mixing error for mesosulfuron-methyl and iodosulfuron-methyl-sodium the 0.5 dose (200 g ha<sup>-1</sup>) was omitted from this herbicide.

Twenty-eight days after treating with herbicide plants were scored for symptoms using a scale with 4 degrees of injury severity: A-no symptoms, B- symptoms

apparent but insufficient to cause plant mortality, C-severe symptoms likely to result in plant mortality, D-complete mortality at time of assessment.

### 2.2.3 Statistical analysis

Based on the four point scale above plants were classified as alive (A and B) or dead (C and D). The proportion of surviving plants per pot was then used as a dependent variable in nonlinear regression (R Development Core Team, 2013; Ritz and Streibig, 2013) for each population by herbicide treatment, fitted to a two parameter log-logistic model of survival:

$$f(x) = \frac{1}{1 + \exp(b(\log(x) - \log(e)))}$$

This function  $f(x)$  gives the proportion of a population which survives exposure to  $x$  herbicide dose, where  $b$  is the slope, and  $e$  the point of inflexion about which the function is symmetric. According to this model, all plants survive zero dose and there is complete mortality at sufficiently high herbicide doses. The symmetrical inflection point therefore gives the LD<sub>50</sub> (lethal dose 50%), the dose at which half of the population are killed. Plant survival is modelled as a binomial response weighted by the total number of individuals in replicate pots. Model fitting was tested using a lack-of-fit test comparing the model against a saturated ANOVA model with each dose treated as a separate group. Model parameters were compared using pair-wise  $T$ -tests.

## 2.3 Results

### 2.3.1 Responses to fenoxaprop-P-ethyl

A lack-of-fit test ( $\chi^2_{44}=33.73$ ,  $P=0.87$ ) indicated that the model fit did not result in a significantly worse fit than a saturated ANOVA model. The lowest doses did not impact survival, with mortality then increasing with increasing dose (fig. 2.2). The 28.46 g a.i. ha<sup>-1</sup> inflexion point of the Oxon population is approximately half the 54.78 g a.i. ha<sup>-1</sup> field rate used, and over 90% control is predicted by the model at this field rate. In contrast the inflexion point for population Berks is at a significantly higher rate of 65.66 g a.i. ha<sup>-1</sup> ( $T=12.04$ ,  $P<0.001$ ).

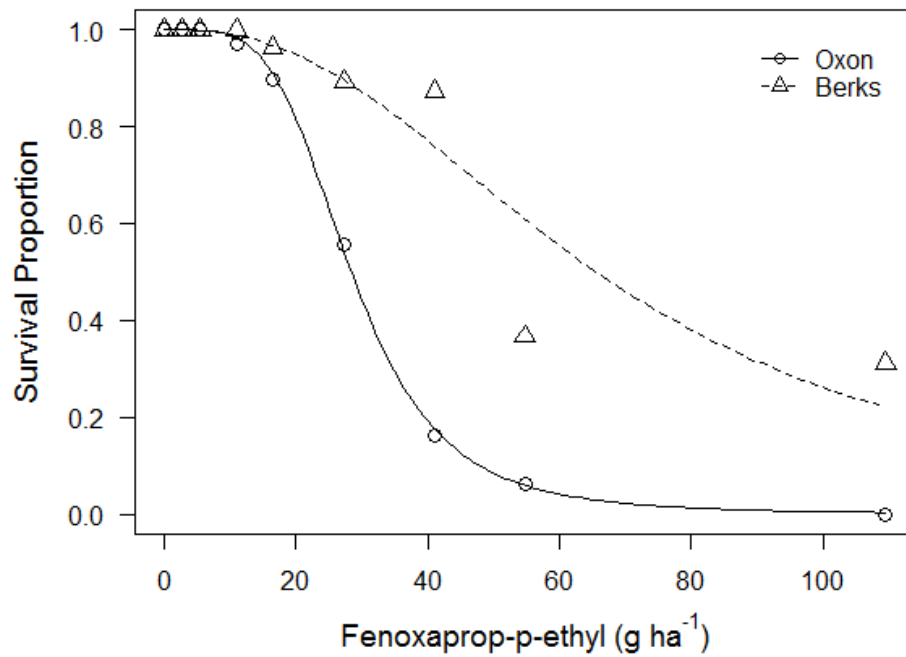


Figure 2.2 Dose response of plant survival four weeks after application of the herbicide fenoxaprop-P-ethyl for two susceptible *A. myosuroides* populations. Symbols are observed mean survival from 3 replicate pots. Lines represent predicted values from 2-parameter log-logistic models.

Table 2.1 Parameters for two parameter log-logistic function describing responses of two susceptible *A. myosuroides* populations based on plant survival four weeks after treating with the herbicide fenoxaprop-P-ethyl.

Parameter	Oxon		Berks	
b (slope)	4.21	(±0.51)	2.46	(±0.36)
e (inflexion point)	28.46	(±1.51)	65.66	(±6.22)

### 2.3.2 Responses to cycloxydim

The lack-of-fit test for responses to cycloxydim was significant ( $\chi^2_{38}=66.82$ ,  $P<0.01$ ) indicating poor model fit. Looking at the responses (fig. 2.3) it can be seen that the doses covered do not show a pattern of declining survival with increasing herbicide dose, but instead have extremely high survival until an abrupt transition to almost complete control, with minor variation in Oxon survival just beyond this threshold likely responsible for the inferior fit compared to saturated ANOVA. Both populations behaved similarly (table 2.2) and there were no significant differences between parameters.

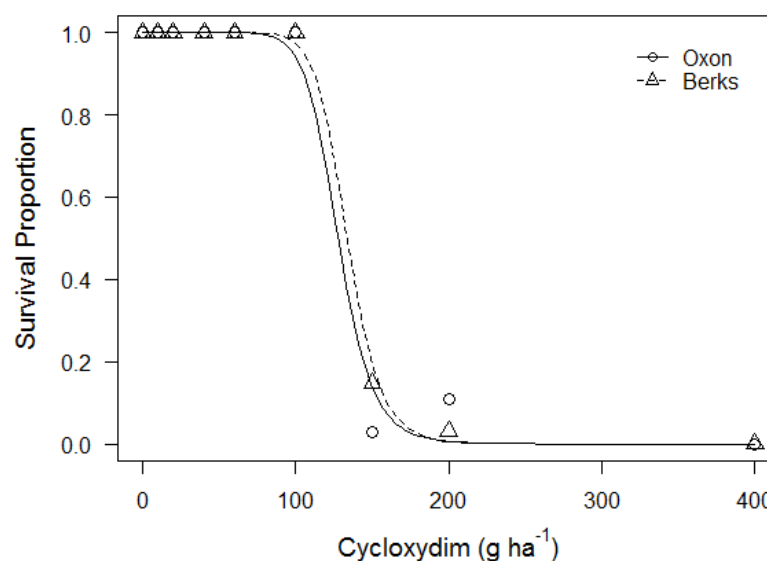


Figure 2.3 Dose response of plant survival four weeks after application of the herbicide cycloxydim for two susceptible *A. myosuroides* populations. Symbols are observed mean survival from 3 replicate pots. Lines represent predicted values from 2-parameter log-logistic models.

Table 2.2 Parameters for two parameter log-logistic function describing responses of two susceptible *A. myosuroides* populations based on plant survival four weeks after treating with the herbicide cycloxydim.

Parameter	Oxon		Berks	
b (slope)	11.38	(±2.40)	12.31	(±3.11)
e (inflexion point)	127.9	(±5.33)	133.9	(±5.35)

### 2.3.3 Responses to mesosulfuron-methyl + iodosulfuron-methyl-sodium

The lack-of-fit test for responses to the mesosulfuron-methyl and iodosulfuron-methyl-sodium herbicide was highly non-significant ( $\chi^2_{38}=21.71$ ,  $P=0.98$ ), indicating good model fit. Both populations behave similarly, with survival rapidly impacted, reaching approximately 90% mortality at the field rate. There were no significant differences between populations for any parameters.

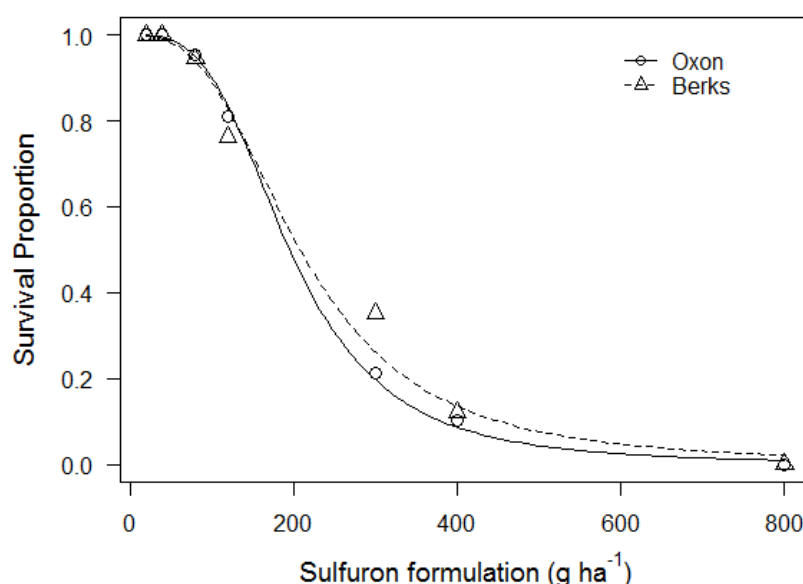


Figure 2.4 Dose response of plant survival four weeks after application of a commercial mesosulfuron-methyl and iodosulfuron-methyl-sodium herbicide mixture for two susceptible *A. myosuroides* populations. Symbols are observed mean survival from 3 replicate pots. Lines represent predicted values from 2-parameter log-logistic models.

Table 2.3 Parameters for two parameter log-logistic function describing responses of two susceptible *A. myosuroides* populations based on plant survival four weeks after treating with a commercial herbicide mixture containing mesosulfuron-methyl and iodosulfuron-methyl-sodium

Parameter	Oxon		Berks	
b (slope)	3.28	(±0.39)	2.84	(±0.39)
e (inflexion point)	195.6	(±14.6)	208.5	(±19.0)

## 2.4 Discussion

### 2.4.1 Variation in response to herbicide application

The conceptual model presented in the introduction described how resistance could be considered as a quantitative trait. The model suggested that greater variance in the underlying quantitative trait distribution for herbicide susceptibility (fig. 2.1b) will reduce the steepness of the slope in the corresponding cumulative frequency distribution (CDF, fig. 2.1a). It was also argued that dose-response assays can broadly be considered as representing the CDF. Subsequently, we may infer that shallow dose response slopes, as observed for fenoxaprop-P-ethyl and mesosulfuron-methyl + iodosulfuron-methyl-sodium, indicate variation in phenotypic response to these herbicides in the populations tested.

In contrast, the very steep slope of the cycloxydim responses suggests there is very little variation in response to this herbicide across the populations tested. The lack of genetic variation in the population results in similar phenotypic resistance for all individuals tested, hence all are controlled above the same dose. From this it might be expected that selection at low doses of cycloxydim from these populations would not result in increasing resistance, as variation is not present to select for and recombine.

The possibility of a low frequency of target-site resistance cannot be discounted, as survival of individual plants in the mortality response assays does not distinguish between mechanisms. Although cycloxydim and fenoxaprop-P-ethyl are both ACCase inhibiting herbicides, individual target-site resistance mutations can result in different levels of resistance to alternative chemical families within the same mode of action (Kaundun et al., 2013b; Kaundun, 2014). However, TSR would not be expected in the numbers of plants tested for plants with no history of herbicide application (as discussed in the introduction). The NTSR mechanisms responsible for low levels of fenoxaprop-P-ethyl resistance may confer some cross-resistance to mesosulfuron-methyl + iodosulfuron-methyl-sodium given the similar shape of their dose-responses, and non-target-site ACCase inhibitor resistance has been shown to potentially confer ALS inhibitor response in *L. rigidum* (Neve and Powles, 2005a). Despite the shared mode of action with fenoxaprop-P-ethyl, this was not the



observed for cycloxydim. Cycloxydim is a poorly metabolisable herbicide (Kaundun et al., 2013a), suggesting that metabolic pathways provided reduced susceptibility to fenoxaprop-P-ethyl in these populations.

The variation in susceptible *A. myosuroides* response to herbicides observed here is perhaps unsurprising. *A. myosuroides* is genetically diverse within populations (Chauvel and Gasquez, 1994; Menchari et al., 2007), and so diversity may be expected in many traits, including herbicide resistance. Though the mechanisms responsible for herbicide survival here are unknown, TSR resistance is unlikely, and so the NTSR brought about by cytochrome P450s and GSTs, or similar mechanisms, are most likely. Cytochrome P450s are an extensive protein family, comprising hundreds of members in plants alone, responsible for important roles in plant defence and stress response (Morant et al., 2003). Similarly, individual plants may contain tens of different GSTs, also involved in stress responses (Frova, 2003). Given the size of these enzyme families, and their wide-ranging functions, it appears likely that within *A. myosuroides* populations genetic variation will be found. Variation in these protein families, and potentially in similar, as yet unconfirmed NTSR mechanisms, could in turn result in the variation in herbicide response observed here.

#### **2.4.2 Levels of survival within susceptible populations**

The frequency of individuals within a susceptible population able to survive field-rates of herbicide would typically be expected to be very low. In the Australian weed *Lolium rigidum* the greatest frequency of survivors following field-rate application of the ACCase inhibiting herbicide diclofop-methyl from an unselected population was 2.6%, while the mean was 0.43% (Neve and Powles, 2005a). These initial survivor frequencies are much higher than expected from mutation rates of major effect genes (discussed in the introduction), and were confirmed to not be caused by TSR mechanisms, suggesting resistance to field-rate herbicide application may be present according to the model presented above. However, in this experiment survivorship at field rate was even greater.

The pre-existing variation in herbicide response may differ between species, according to the genetic architecture of the trait, and intrinsic differences in herbicide selectivity, but the apparent very high initial resistance observed here may in part be

a result of experimental conditions. Plants were sown relatively late (compared to typical agricultural winter wheat season), and germination and early growth took place in the unusually cold December of 2010 (Met Office, 2011), which may have resulted in cold acclimation. Cold acclimation has a broad response in plants, and has been demonstrated in *Arabidopsis* to increase tolerance of oxidative stress resulting from herbicides (Bridger et al., 1994) and activate multiple regulatory pathways, including glutathione *S*-transferases (Fowler and Thomashow, 2002), known to be involved in NTSR (Powles and Yu, 2010; Yuan et al., 2007) and potentially especially important in providing resistance in *A. myosuroides* (Cummins et al., 2011, 2013). Even if low temperatures had not resulted in survival due to cold acclimation they could have resulted in over-estimation by slowing plant growth. Only when cells are actively dividing as the plant grows will the systemic herbicides applied in this experiment act.

Slow growth may also have led to underestimation of mortality by masking the difference between healthy plants that had only grown a small amount and plants that had not grown at all. Even under normal growth, the 4 point injury scale may have incorrectly defined some plants as survivors if there was no visible damage to the plant apparent at the point of assessment. Aside from potentially colder conditions immediately following germination, the experiment presents a much less stressful environment for plants than field conditions. In the field weeds would be expected to be under competition with the crop and other weeds, and potentially face greater exposure to pests and environmental uncertainty, and subsequently may have been less able to survive exposure to herbicide. The high levels of survival at field rate in this experiment will therefore not necessarily be observed in the field.

The highest level of resistance was observed in the Berks population responding to fenoxaprop-P-ethyl. Mean survival was greater for Berks than Oxon at all doses (fig. 2.2). This may reflect the different recent history of the two populations. The Berks population was obtained directly from an organic farm, which may have had gene inflow via pollen or seed movement from *A. myosuroides* populations in the surrounding area that were exposed to herbicides. In comparison the Oxon population has been grown in dedicated production beds since originally being harvesting in 2004. Confirmed cases of herbicide resistance in *A. myosuroides* in the UK have increased from “over 700” farms in 1999 (HGCA, 1999) to “over 16000”

in 2010 (Moss, 2013a). While these figures undoubtedly reflect differences in awareness and testing for resistance, and both almost certainly greatly underestimate the real incidences of *A. myosuroides* resistance, the difference suggests a large increase in resistant *A. myosuroides* over the last 15 years.

Dose-responses are inherently difficult to replicate, with a range of environmental factors influencing results (Medd et al., 2001). Despite some hesitation concerning the exact levels of resistance found, a clear variation in response to herbicide was shown in this experiment. Building on this experience future dose-responses were performed with some revisions (chapter 3.2).

### **2.4.3 Implications for selection for herbicide resistance**

The dose-responses performed suggested that there was a great deal of variation in the responses to fenoxaprop-P-ethyl and mesosulfuron-methyl + iodosulfuron-methyl-sodium for both susceptible populations tested. While there are some concerns that survival may have been over-estimated, this reflects a systematic problem occurring at all doses, which would not be expected to significantly change the overall shape of the curve. If this variation is heritable, selecting within the pre-existing variation in herbicide response would be expected to rapidly evolve enhanced resistance by selecting for and recombining the alleles responsible, increasing the mean of the quantitative trait. Conversely, the steep threshold seen in the cycloxydim dose-responses suggests that plants in both populations respond very similarly to cycloxydim, and so there is not a background of additive genetic variation to select from and increase the phenotypic resistance trait.

The ability to select for increased resistance from within this pre-existing variation is tested in chapter 3 using the Oxon population selecting with low-doses of fenoxaprop-P-ethyl.

### **3. Evolution of herbicide resistance in *Alopecurus myosuroides* by low-dose fenoxaprop-P-ethyl selection within standing genetic variation**

#### **3.1 Introduction**

In order to account for the extremely rapid evolution of herbicide resistance in weedy plants the initial stages of herbicide selection must be understood (Neve et al., 2009). As discussed in sections 1.2 and 2.1, the two major categories of herbicide resistance; target-site and non-target site (TSR and NTSR respectively); are thought to differ not only in their resistance conferring mechanisms, but also in their underlying genetic architecture, and hence how they may be selected. There is increasing evidence that NTSR is a polygenic trait (Délye, 2013). As such, pre-existing variation in NTSR mechanisms may be observed as a quantitative phenotypic response in herbicide dose assays conducted on naïve weed populations (those with no history of herbicide exposure), as demonstrated in chapter 2. If variation in phenotypic resistance arises from additive genetic variation, selection with herbicides at appropriate doses (within the range of phenotypic variation) will result in population-level responses to selection and increases in the mean phenotypic value for resistance. Predicting responses to selection on additive genetic variation is possible if the genetic architecture and heritability of the trait are known. In the absence of this information, selection experiments can be performed to determine responses to selection.

The potential for selection for herbicide resistance to occur from pre-existing additive genetic variation depends upon how the selection pressure imposed relates to the level of phenotypic variation within a population (fig. 3.1), as originally argued for insecticide resistance (McKenzie, 2000). If genetic variation gives rise to variation in herbicide resistance phenotype, lower herbicide doses may select for increased resistance within this standing genetic variation (fig. 3.1a). In outcrossing species, selection and recombination of additive genetic variation may rapidly increase resistance in subsequent generations. At higher doses, beyond the pre-existing range of phenotypic variation, the only means of survival is via novel, major effect mutations providing strong phenotypic resistance (fig. 3.1b).

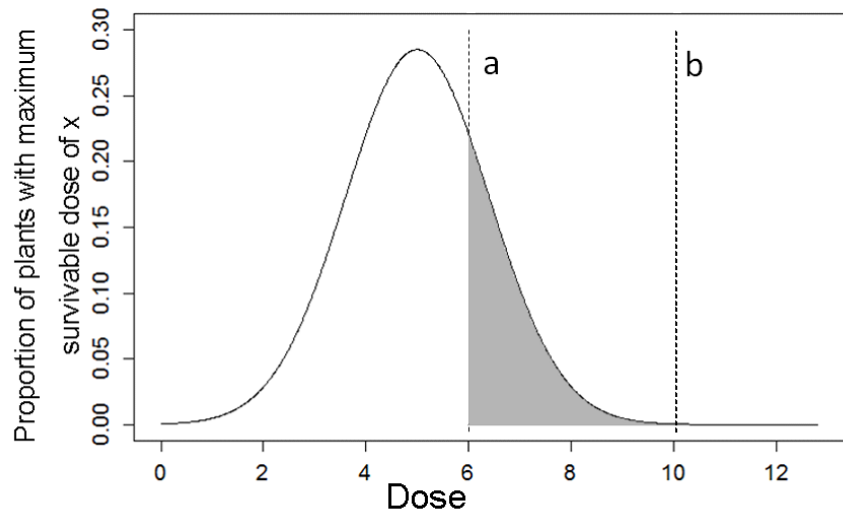


Figure 3.1 A hypothetical population for which maximum survivable herbicide dose is a normally distributed quantitative trait of mean 5 (arbitrary units). Applying a dose of 6 units (a) is within the pre-existing phenotypic trait distribution, and a proportion of individuals can survive (shaded grey), selecting for increased resistance from standing genetic variation. Applying a dose of 10 units (b) is outside the range of pre-existing phenotypic variation and would be expected to kill all individuals.

The degree to which a quantitative trait can shift from one generation can be considered using the breeder's equation (Lush, 1943):

$$\Delta Z = h^2 S \text{ (eqn. 3.1)}$$

where  $\Delta Z$  is the change in mean trait  $Z$  between generations,  $S$  the selection differential, and  $h^2$  the heritability of the trait in question. In its simplest form under truncation selection, the selection differential is the difference between the mean phenotypic value of the parent population and the mean phenotypic value of selected individuals (Falconer et al., 1996). In selection for herbicide resistance, the selected individuals are those that survive a given herbicide dose. The degree to which this selection differential then results in changes to the trait distribution in the next generation is determined by heritability. Heritability is defined as the proportion of phenotypic variation attributable to genetic variance for a given trait (Lynch and Walsh, 1998). Both the heritability of a trait, and the pre-existing phenotypic variation from which a selection differential is established, depend on the underlying genetic architecture: how much genetic variation exists and the nature of any

interactions between contributing alleles (McGuigan and Sgrò, 2009; Le Rouzic and Carlborg, 2008). Depending upon the population genetic structure for the trait in question, the size of the population subsamples exposed to selection may reduce the genetic variation for that trait, and hence limit the response to selection.

The question of whether adaptation primarily occurs from quantitative standing genetic variation or via major effect mutations is not unique to herbicide resistance, and reflects ongoing debate in broader evolutionary ecology (Orr, 2005; Barrett and Schluter, 2008). An understanding of the potential for dose rate to influence the evolution of resistance is especially important to consider for resistance management. It has been suggested that as legislation to limit herbicide use is introduced, the lower doses used will accelerate the evolution of resistance (Gressel, 2009). However, high doses will very rapidly select for any major effect mutations that arise (Renton et al., 2011), leading to what might be considered a ‘lose-lose’ scenario where resistance is selected for by both high and low dose strategies (Gressel, 2002). These dynamics may also occur in a two-step process, whereby minor gene quantitative resistance gradually increases population sizes, increasing mutation supply and subsequently the potential for a major effect mutation to arise (Neve et al., 2009). The likelihood and consequences of this process occurring depend upon how rapidly resistance can evolve from standing genetic variation, and the frequency of TSR mutations. The impact of dose strength has also been considered on the evolution of resistance to other pesticides. In insecticides similar conceptual models have been proposed, suggesting the insecticide dose applied determines which resistance mechanisms are selected (ffrench-Constant et al., 2004), while in fungicides most studies have found that selection at higher doses is most likely to result in the evolution of resistance (van den Bosch et al., 2011). Concerns over the impact of dose strength on resistance evolution have also been raised for microbial resistance to antibiotics (Hughes and Andersson, 2012), but debate continues regarding the scale and implications of these effects (Andersson and Hughes, 2014).

Understanding the dynamics for initial resistance selection may also present opportunities for resistance management (Neve et al., 2009). If herbicide application is removed for a generation, selection for resistance will be relaxed. If resistance is associated with pleiotropic fitness costs, relaxation of selection will select against

resistance (Lahti et al., 2009). Relaxation of selection may produce complex dynamics, however, and may even increase the phenotypic trait under selection, as reported for parasite resistance in the guppy (*Poecilia reticulata*) (Dargent et al., 2013). Costs of resistance are discussed further in chapter 4, and resistance management strategies varying selection pressure are considered in chapter 5.

Characterisation of field resistance has frequently identified major effect genes, conferring resistance through target-site mutations, and suggested this was the primary means by which field resistance evolved (Jasieniuk et al., 1996). More recently, the potential for low doses to select within pre-existing variation in herbicide response and increase herbicide resistance has been considered in terms of the theoretical model presented above. The concept was first demonstrated in *Lolium rigidum*, where recurrent selection with low doses of the ACCase inhibiting herbicide diclofop-methyl resulted in the rapid evolution of resistance to the herbicide at much higher doses (Neve and Powles, 2005b; Busi et al., 2013a), and has also been confirmed under field conditions (Manalil et al., 2011). Recurrent selection at low herbicide doses has also been shown to result in resistance evolution in *L. rigidum* for the EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) inhibiting herbicide glyphosate (Busi and Powles, 2009) and the very-long-chain fatty acid synthesis inhibiting (Tanetani et al., 2009) herbicide pyroxasulfone (Busi et al., 2012). Despite its importance as an agricultural pest, and its high propensity to evolve herbicide resistance, these effects of sub-lethal herbicide selection have not previously been tested in *A. myosuroides*.

In this chapter selection is imposed within pre-existing variation in herbicide response in a susceptible *A. myosuroides* population to determine whether herbicide resistance can evolve. Five key hypotheses for the early stages of selection for herbicide resistance are tested: (i) Selection using sub-lethal herbicide doses will increase the phenotypic resistance profile in progeny. (ii) Responses to selection will vary according to the selection differential (two different herbicide doses are tested). (iii) Responses to selection will depend on population size (three different population sizes are tested). (iv) Recurrent selection will continue to increase the phenotypic resistance profile, following selection with either the same herbicide dose or an increased dose. (v) Relaxed selection will result in a reduction of the phenotypic resistance profile.

## **3.2 Materials and methods**

### **3.2.1 Plant material**

The ‘Oxon’ population described in section 2.2.1 was the susceptible stock population from which all selection experiments below were performed.

### **3.2.2 Initial fenoxaprop-P-ethyl selection**

To establish the potential for selection within the range of phenotypic variation in herbicide response to result in resistance evolution, susceptible *A. myosuroides* was exposed to two different doses: 13.6 and 27.2g a.i. ha<sup>-1</sup> of commercial fenoxaprop-P-ethyl. These doses represent approximately 25% and 50% of the recommended field dose of 54.78g a.i. ha<sup>-1</sup> used in chapter 2, and were selected based on results in figure 2.2. Three different population sizes, 100, 200 and 600 individuals, were exposed to each dose. These groups are subsequently referred to by the population size exposed (n), followed by the dose received (d), e.g. “n=200, d=27.2” to describe the progeny following selection at 27.2g a.i. ha<sup>-1</sup> from 200 individuals. In April 2011 seeds were sown in 180-cell (2x2x5 cm cells) grower’s trays filled with ‘J Arthur Bower’s top-soil’ (1 tray for groups testing 100 individuals, 2 for 200, and 6 for 600). Seeds were double sown with 2 seeds planted in each cell. Trays were maintained in a polythene tunnel and watered daily, arranged in a completely randomised layout.

In May 2011 plants were thinned so that pooled trays contained the appropriate number of individuals, with all plants at the 2-3 leaf stage and no more than one plant per cell. Herbicide was then applied as described in section 2.2.2.

28 days after spraying plants were assessed for survival. Survival was confirmed by evidence of continued plant growth following herbicide application. Surviving plants were carefully removed from the growing trays, leaves were trimmed to 2cm and roots to 5cm, and plants were re-potted in 15cm diameter pots filled with top-soil. Four surviving plants were placed in each 15cm pot. All pots for each population size by dose treatment were then isolated within individual compartments within a polythene tunnel to enable bulk-crossing between surviving plants. Pots were watered as required and a bulk seed collection was made at maturity for each population size by dose combination. Following harvesting seeds were threshed,



cleaned and dried at 15°C, 15% relative humidity and subsequently stored at 5°C in hermetic heat-sealed foil pouches until required.

### **3.2.3 Recurrent fenoxaprop-P-ethyl selection**

Seed population  $n=200$ ,  $d=27.2$  was used to test whether recurrent selection could further increase resistance profile. This seed population was used as a clear increase in mean resistance was observed in a preliminary dose-response following the first round of selection (data not shown, but see section 3.3.2 below for repeated dose-response results). Recurrent selection was performed with the same initial population size of 200 individuals, and tested using the same herbicide dose, 27.2g a.i. ha<sup>-1</sup>, and an increased dose of 48g a.i. ha<sup>-1</sup>. These selection lines follow the naming convention described above, with the second dose following after a comma, e.g. “ $n=200$   $d=27.2$ , 27.2”. In January 2012 the seeds harvested from the first generation of selection underwent a dormancy breaking treatment of 3 weeks in the dark at 35°C in dry conditions. In February 2012 seeds were sown in grower’s trays and maintained in a polythene tunnel, arranged in a completely randomised layout, as described in section 3.2.2. In March 2012 plants were thinned so that pooled trays contained 200 individuals at the 2-3 leaf stage and no more than one plant per cell. Herbicide was then applied as described in chapter 2.2.2.

28 days after spraying plants were assessed, replanted in 15cm diameter pots, and moved to separate enclosures for each dose, as described in section 3.2.2. Plants were maintained in separate enclosures from March-September 2012. At the end of September 2012 seeds were harvested, cleaned and stored as described in section 3.2.2

### **3.2.4 Relaxation of fenoxaprop-P-ethyl selection**

Using the same  $n=200$ ,  $d=27.2$  population, a generation with no herbicide selection was tested to determine whether relaxation of selection would reduce phenotypic resistance. This experiment was carried out at the same time as the recurrent selection, with seeds sown in grower’s trays in February 2012 and maintained in a polythene tunnel, included in the completely randomised layout of plants described in section 2.3. In February 2012, plants were thinned so that only 200 plants at the 2-3 leaf growth stage remained. In March 2012 leaves were trimmed to 2cm and roots

to 5cm for these 200 plants, and they were re-potted in 15cm diameter pots filled with top-soil, with 4 plants per pot, to match conditions of herbicide selected groups. Pots were then maintained in an individual enclosure within a polythene tunnel from March-September in order to bulk cross. At the end of September 2012 seeds were harvested, cleaned and stored as described in section 3.2.2.

### **3.2.5 Quantification of herbicide responses**

Following the completion of two rounds of low-dose selection, a series of dose response experiments were performed with fenoxaprop-P-ethyl to determine the response to selection. Dose-responses were performed for all selection lines described above, including relaxed selection, and for the original ‘Oxon’ population (table 3.1). Dose-responses for all groups were performed at the same time to control for differences in herbicide application and growth conditions.

In January 2013 seeds from all lines described above underwent a dormancy breaking treatment of 3 weeks at 35°C in the dark in dry conditions. Seeds were then sown at 1.5cm depth in 21 equally spaced locations in 15cm diameter pots filled with 50% ‘J Arthur Bowers Top-Soil’, 25% Levington M2 compost and 25% sand. At each location two seeds were sown at a depth of 1.5cm and pots were re-covered with the soil mixture. Pots were maintained in a glasshouse with 14 hour photoperiods (with supplementary lighting to achieve this), heated to 20°C in the day and 18°C at night. Pots were arranged in three completely randomised replicate blocks and watered daily. In February 2013 plants were thinned so that each sown location contained only a single plant at the 1.5-3 leaf stage and herbicide was applied at a range of doses. The doses differed for each selection line (table 3.1), with doses selected to cover the breadth of their dose-response profile following preliminary dose-responses performed in December 2012 (data not shown). Herbicide application was performed as described in chapter 2.2.2, and pots were returned to the same glasshouse conditions. Two separate measures of plant response to herbicide were taken: mortality and fresh weight of individual plants. 21 days after herbicide application all plants (regardless of visible herbicide symptoms) were cut to 1cm from the soil surface and fresh weight measured individually. After a further 21 days plant survival was scored. Plants were scored as alive if there had been regrowth of fresh leaf material.

Table 3.1 Fenoxaprop-P-ethyl dose-responses for each selection line. Selection lines are referred to by the population size selected from, n, followed by the fenoxaprop-P-ethyl dose or doses applied, d (g a.i. ha<sup>-1</sup>).

<i>A. myosuroides</i> group	Herbicide doses tested (g a.i. ha <sup>-1</sup> )
Original population	0, 6.8, 13.6, 34, 68, 136
n=100, d=13.6	0, 6.8, 13.6, 34, 68, 272
n=200, d=13.6	0, 6.8, 13.6, 34, 68, 272
n=600, d=13.6	0, 6.8, 13.6, 34, 68, 272
n=100, d=27.2	0, 6.8, 13.6, 34, 68, 272
n=200, d=27.2	0, 6.8, 13.6, 34, 68, 272
n=600, d=27.2	0, 6.8, 13.6, 34, 68, 272
n=200, d=27.2, 27.2	0, 13.6, 34, 68, 136, 272
n=200, d=27.2, 48	0, 13.6, 34, 68, 136, 272
n=200, d=27.2, 0	0, 6.8, 13.6, 34, 68, 272

### 3.2.6 Statistical analysis

For survival data a two parameter log-logistic model was fitted, as described in section 2.2.3. For fresh weight data, similar analysis was performed using a four parameter log-logistic function:

$$f(x) = c + \frac{d-c}{1+\exp(b(\log(x)-\log(e)))} \text{ (eqn. 3.2)}$$

The function  $f(x)$  gives the expected fresh biomass of plants following exposure to  $x$  herbicide dose. As in the two parameter model, parameter  $b$  is the slope, and  $e$  the inflexion point. The  $d$  parameter estimates the fresh biomass at dose 0, i.e. the weight of plants that did not receive herbicide application, and  $c$  the minimum biomass. In a four parameter log-logistic function the inflexion point is midway between these upper and lower limits, hence  $e$  gives the ED<sub>50</sub> (effective dose 50%, see section 2.2.3 for comparison to LD<sub>50</sub>). Model fits were tested using a lack-of-fit test which compares the model against an ANOVA with each dose as a separate group. Model parameters were compared to each other using pair-wise  $T$  tests: where ED<sub>50</sub> or LD<sub>50</sub> R:S ratios are described, significance levels reported are  $T$  test comparisons of resistant and susceptible ED<sub>50</sub> or LD<sub>50</sub> parameter estimates.

### 3.3 Results

#### 3.3.1 Original population ('Oxon')

The mortality dose response for the original population (fig. 3.3a, table 3.2) shows that at the field rate of 55 g ha<sup>-1</sup> there is complete control, with the variation in herbicide responses only evident at lower doses. The fresh-weight dose response was similar (fig. 3.3b), with severe reductions in fresh weight observed at the field rate. In all following results comparing resistant to susceptible parameters, a resistance index is calculated as the ratio of the LD<sub>50</sub> or ED<sub>50</sub> of the selected line to the original, unselected population. In these R:S resistance indices the selected line is always the 'resistant' R population, even if there is no increase in resistance, and the 'susceptible' S population is always the original population.

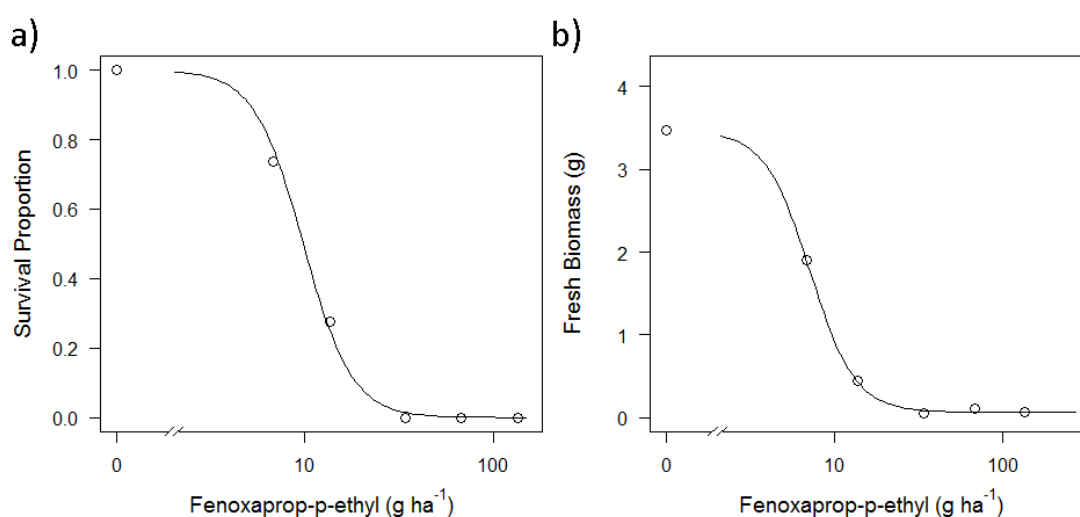


Figure 3.2 Dose-response curves for an *A. myosuroides* population following application of fenoxaprop-P-ethyl at the two to three leaf stage. Responses are a) mortality scored via plant regrowth and b) Fresh above ground biomass 21 days after herbicide application. Lines are predicted values of the dose-response models, symbols are mean values at each dose tested.

Table 3.2 Estimates and standard errors for LD<sub>50</sub> in fenoxaprop-P-ethyl dose-mortality responses of a susceptible black-grass populations and lines derived from herbicide selection

<i>A. myosuroides</i> line	LD <sub>50</sub>	LD <sub>50</sub> R/S
Original population	9.78 (±1.10)	
100 (13.6)	13.12 (±2.38)	1.34
200 (13.6)	16.30 (±2.07)	1.67
600 (13.6)	18.42 (±4.43)	1.88
100 (27.2)	10.78 (±1.17)	1.10
200 (27.2)	49.25 (±9.94)	5.04
600 (27.2)	21.25 (±2.76)	2.17
200 (27.2, 27.2)	55.42 (±9.21)	5.67
200 (27.2, 48)	59.32 (±7.90)	6.07
200 (27.2, 0)	19.95 (±3.21)	2.04

Table 3.3 Estimates and standard errors for fenoxaprop-P-ethyl biomass dose-responses of a susceptible black-grass populations and lines derived from herbicide selection

<i>A. myosuroides</i> line	ED <sub>50</sub>	<i>d</i> (max)	<i>c</i> (min)	ED <sub>50</sub> R/S
Original population	7.13 (±0.47)	3.47 (±0.17)	0.06 (±0.10)	
100 (13.6)	11.80 (±2.31)	2.41 (±0.14)	0.04 (±0.11)	1.65
200 (13.6)	11.80 (±1.58)	2.42 (±0.18)	0.01 (±0.13)	1.65
600 (13.6)	7.79 (±0.64)	3.84 (±0.25)	0.10 (±0.09)	1.09
100 (27.2)	7.46 (±0.52)	3.00 (±0.17)	0.03 (±0.10)	1.05
200 (27.2)	11.52 (±2.89)	2.26 (±0.15)	0.12 (±0.20)	1.62
600 (27.2)	13.14 (±1.03)	1.92 (±0.13)	0.07 (±0.09)	1.84
200 (27.2, 27.2)	19.55 (±2.80)	2.32 (±0.16)	0.13 (±0.12)	2.74
200 (27.2, 48)	13.12 (±3.32)	2.22 (±0.13)	0.03 (±0.17)	1.84
200 (27.2, 0)	10.98 (±1.01)	2.95 (±0.18)	0.12 (±0.09)	1.54

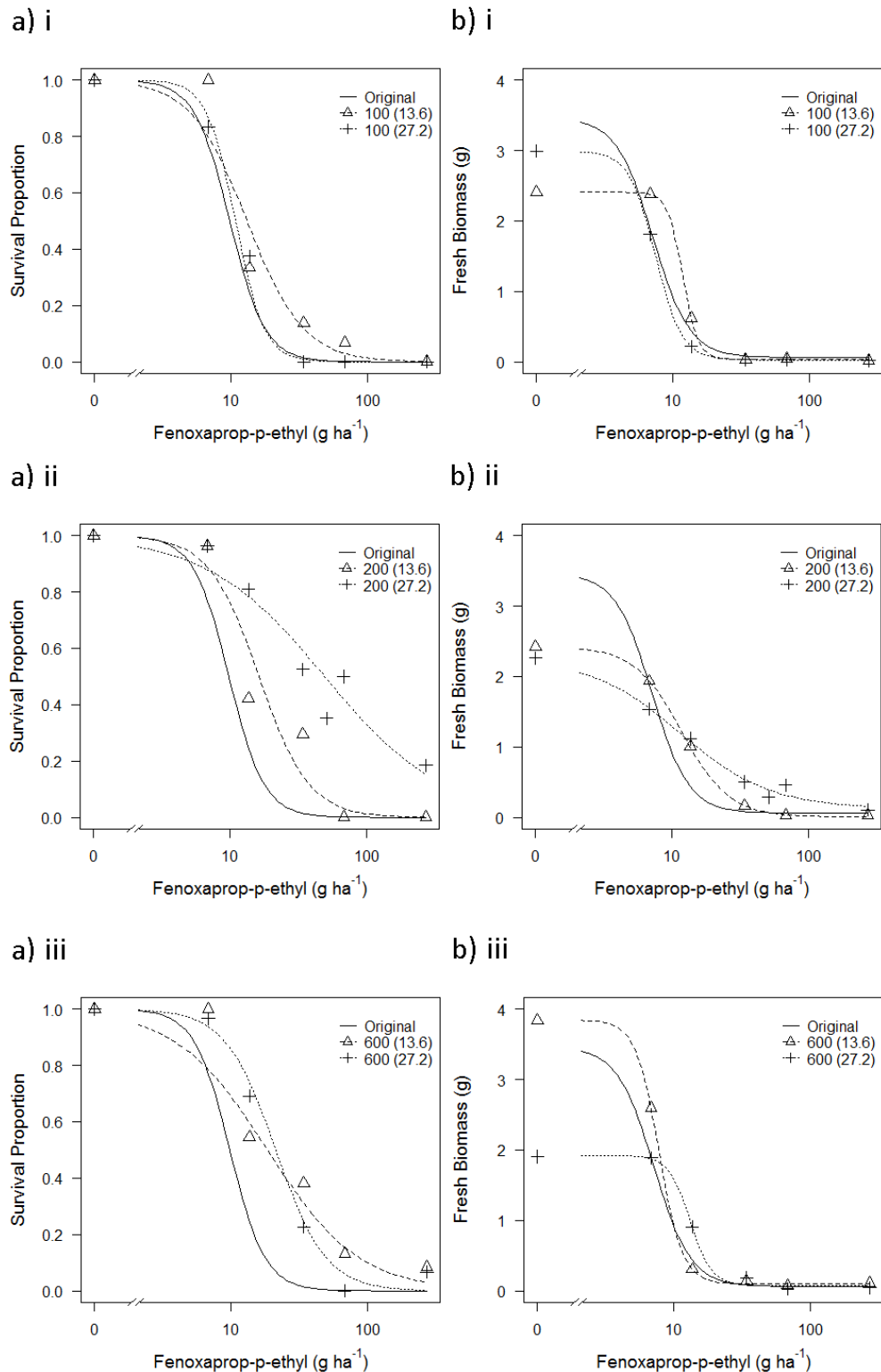


Figure 3.3 Fenoxaprop-P-ethyl a) mortality and b) above ground fresh biomass (21 days after herbicide application) dose-response curves for *A. myosuroides* populations following selection for survival of 13.6 and 27.2 g ha<sup>-1</sup> fenoxaprop-P-ethyl from i) 100 individuals, ii) 200 individuals and iii) 600 individuals. Lines are predicted values of the dose-response models, symbols are mean values at each dose tested. Datapoints for original population (shown in fig.3.2) omitted for clarity.

### 3.3.2 First generation of selection – effects of herbicide dose and population size

#### i) *Selection from 100 individuals*

Selection from populations of  $n=100$  did not significantly change the  $LD_{50}$  inflexion point, parameter  $e$ , following selection at  $d=13.6$  ( $T=1.60$ ,  $P=0.11$ ) or  $d=27.2$  ( $T=0.65$ ,  $P=0.52$ ) (fig. 3.4 a,i). In the fresh-weight responses (fig. 3.4 b,i)  $n=100$ ,  $d=13.6$  resulted in an  $ED_{50}$  of  $11.80 \text{ g ha}^{-1}$  ( $\pm 2.3$ ), significantly greater than the original population ( $ED_{50}$  R:S ratio=1.65;  $T = 3.16$ ,  $P = 0.002$ ); in contrast to  $n=100$ ,  $d=27.2$ , which remained similar to the original population ( $ED_{50}$  R:S ratio=1.05;  $T = 0.47$ ,  $P = 0.64$ ). Fresh weight of both  $n=100$ ,  $d=13.6$  and  $n=100$ ,  $d=27.2$  was significantly lower than the unselected population in the absence of herbicide application ( $T = 4.02$ ,  $P < 0.001$  and  $T = 2.96$ ,  $P=0.003$ , respectively).

#### ii) *Selection from 200 individuals*

Both  $n=200$ ,  $d=13.6$  and  $n=200$ ,  $d=27.2$  showed significant responses to selection, with  $LD_{50}$  R:S ratios of 1.67 ( $T = 3.92$ ,  $P < 0.001$ ) and 5.04 ( $T = 17.5$ ,  $P < 0.001$ ) respectively (fig. 3.4 a,ii). The increase in  $LD_{50}$  was significantly greater following selection with the higher dose,  $d=27.2$  than  $d=13.6$  ( $T=8.48$ ,  $P<0.001$ ). Increased resistance was also observed for fresh weights (fig. 3.4 b,ii), with both selection lines displaying a significant increase in  $ED_{50}$  (for  $n=200$ ,  $d=13.6$ :  $T=4.39$ ,  $P<0.001$ ; for  $n=200$ ,  $d=27.2$ :  $T=0.47$ ,  $P<0.001$ ), but there was not a significant difference in  $ED_{50}$  between selection with either doses ( $T=0.08$ ,  $P=0.9$ ). As observed for the  $n=100$  groups, both  $n=200$ ,  $d=13.6$  and  $n=200$ ,  $d=27.2$  also displayed a significant reduction in fresh biomass in the absence of herbicide ( $T = 3.40$ ,  $P < 0.001$  and  $T = 4.21$ ,  $P < 0.001$  respectively).

#### iii) *Selection from 600 individuals*

Significant increases in  $LD_{50}$  were observed for both  $n=600$ ,  $d=13.6$  (R:S=1.88,  $T = 3.33$ ,  $P<0.001$ ) and  $n=600$ ,  $d=27.2$  (R:S=2.17,  $T = 6.82$ ,  $P<0.001$ ) (fig. 3.4 a,iii). The increases in  $LD_{50}$  did not differ between selection with either dose ( $T=0.56$ ,  $P=0.57$ ). In the fresh weight response curves (fig. 3.4 b iii) only the higher dose group,  $n=600$ ,  $d=27.2$ , showed a significant increase in  $ED_{50}$  (R:S = 1.84,  $T = 8.20$ ,  $P < 0.001$ ). In  $n=600$ ,  $d=27.2$  a significant reduction in weight in the absence of herbicide was also

observed ( $T = 5.30$ ,  $P < 0.001$ ), while  $n=600$ ,  $d=13.6$  did not result in any reduction in biomass (with a non-significant increase instead observed).

Selection at  $d=13.6$  resulted in similar  $LD_{50}$  increases for  $n=200$  and  $n=600$  ( $T = 0.48$ ,  $P = 0.63$ ), but selection at  $d=27.2$  resulted in a significantly greater  $LD_{50}$  for  $n=200$  than  $n=600$  ( $T = 2.37$ ,  $P = 0.02$ ).

### 3.3.3 Recurrent fenoxaprop-P-ethyl selection

A second generation of selection for  $n=200$ ,  $d=27.2$ , at the same dose of  $27.2 \text{ g ha}^{-1}$  (resulting in the population denoted  $n=200$ ,  $d=27.2$ ,  $27.2$ ) resulted in a significantly increased  $LD_{50}$  compared to the original population ( $T=23.2$ ,  $P<0.001$ ), but did not represent a further significant increase above  $n=200$ ,  $d=27.2$  ( $T=0.85$ ,  $P=0.40$ ) (fig. 3.5a). The same trend was observed in fresh weight responses (fig. 3.5 b), with a significantly increased  $ED_{50}$  in comparison to the original population ( $T=11$ ,  $P<0.001$ ) which did not significantly differ from  $n=200$ ,  $d=27.2$  ( $T=1.42$ ,  $P=0.16$ ). There were indications that recurrent selection at  $27.2 \text{ g ha}^{-1}$  resulted in increased tolerance at lower herbicide doses not reported by the main dose-response parameters, with  $n=200$ ,  $d=27.2$ ,  $27.2$  estimating  $ED_{30}$  (the dose at which individual plant weight is expected to decrease by 30%) of  $14.1 \text{ g ha}^{-1}$  ( $\pm 2.22$ ), much greater than that predicted for  $n=200$ ,  $d=27.2$  ( $6.03 \pm 1.61$ ) and original ( $5.49 \pm 0.53$ ) population.

Recurrent selection, first at  $27.2 \text{ g ha}^{-1}$  then at an increased dose of  $48 \text{ g ha}^{-1}$  (resulting population denoted  $n=200$ ,  $d=27.2$ ,  $48$ ), showed a similar response to  $n=200$ ,  $d=27.2$ ,  $27.2$ . For  $n=200$ ,  $d=27.2$ ,  $48$  the  $LD_{50}$  ( $T=29.1$ ,  $P<0.001$ ) and  $ED_{50}$  ( $T=3.21$ ,  $P=0.001$ ) were significantly greater than the original population, but did not represent significant increases beyond  $n=200$ ,  $d=27.2$  ( $LD_{50}$  :  $T=0.85$ ,  $P=0.40$ ;  $ED_{50}$  :  $T=0.34$ ,  $P=0.73$ ).



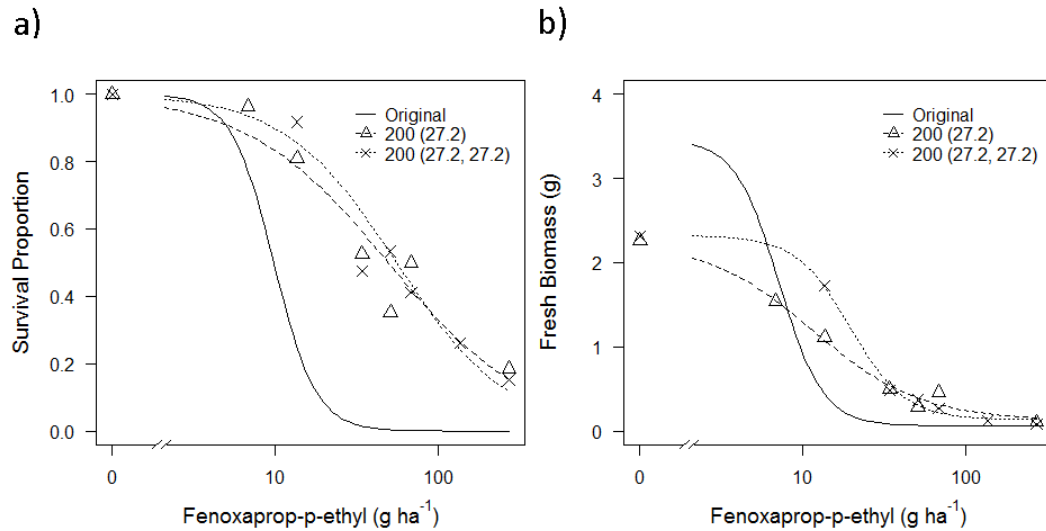


Figure 3.4 Fenoxaprop-P-ethyl a) mortality and b) fresh-weight dose-response curves for *A. myosuroides* populations following selection for a single generation and recurrent selection for two generations at  $27.2 \text{ g ha}^{-1}$  fenoxaprop-P-ethyl from 200 individuals. Lines are predicted values of the dose-response models, symbols are mean values at each dose tested.

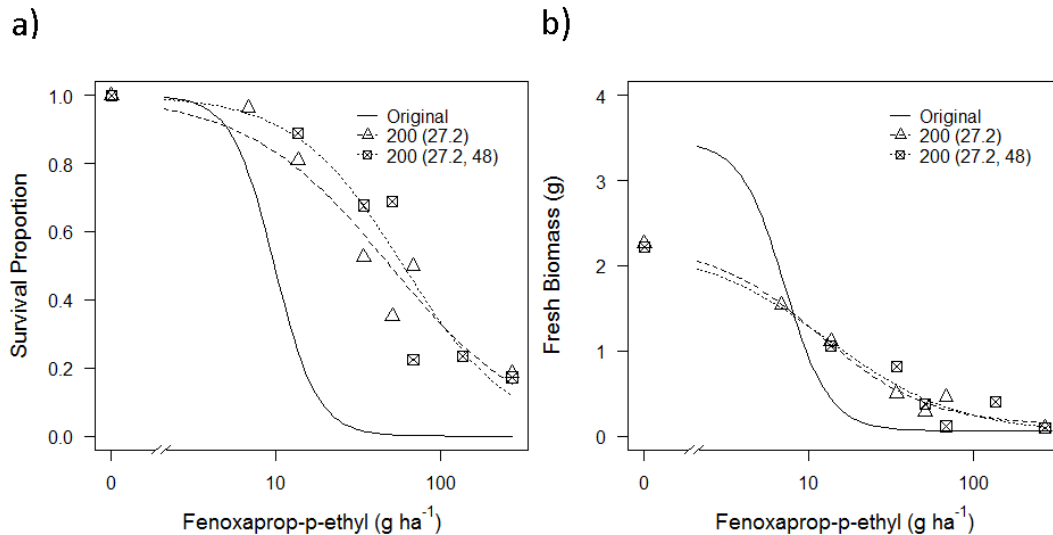


Figure 3.5 Fenoxaprop-P-ethyl a) mortality and b) fresh-weight dose-response curves for *A. myosuroides* populations following selection for a single generation at  $27.2 \text{ g ha}^{-1}$  fenoxaprop-P-ethyl and selection for two generations at a dose of  $27.2$  followed by  $48 \text{ g ha}^{-1}$  fenoxaprop-P-ethyl. Lines are predicted values of the dose-response models, symbols are mean values at each dose tested.

### 3.3.4 Relaxation of fenoxaprop-P-ethyl selection

Following selection at  $27.2 \text{ g ha}^{-1}$  by a generation without any selection (resulting population denoted  $n=200, d=27.2, 0$ ) significantly decreased  $LD_{50}$  compared to  $n=200, d=27.2$  ( $T=2.31, P=0.02$ ). However, the  $LD_{50}$  for  $n=200, d=(27.2, 0)$  was still significantly greater than the original population ( $R:S = 2.04, T = 5.30, P < 0.001$ ). For fresh weight responses,  $n=200, d=27.2, 0$  showed significantly greater  $ED_{50}$  than the original population ( $T=4.77, P<0.001$ ), but it did not significantly differ from  $n=200, d=27.2$  ( $T=0.19, P=0.852$ ). Fresh weight in the absence of herbicide was significantly greater for  $n=200, d=27.2, 0$  than  $n=200, d=27.2$  ( $T=2.57, P=0.01$ ), and fresh weight in the absence of herbicide for  $n=200, d=27.2, 0$  was not significantly less than the original population ( $T=1.89, P=0.06$ ).

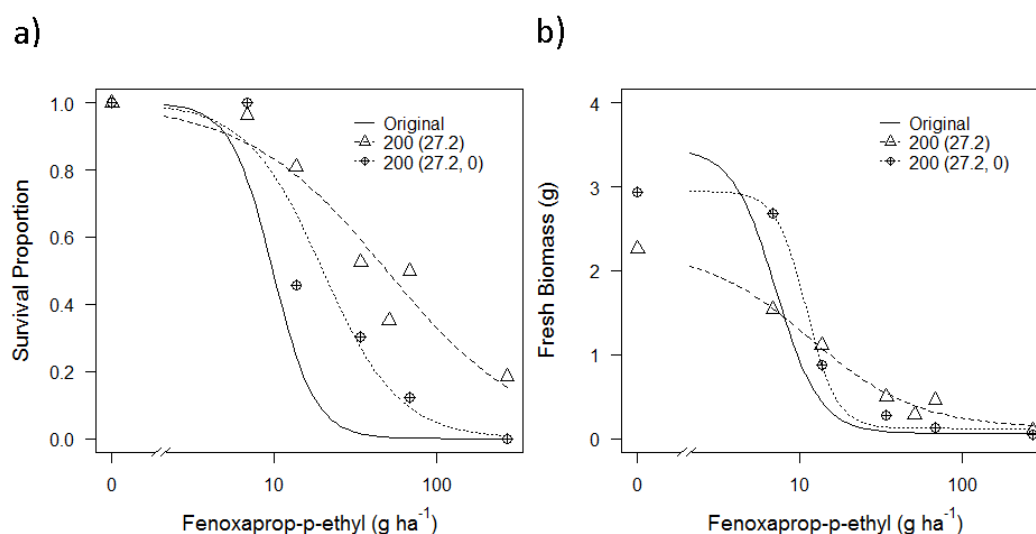


Figure 3.6 Fenoxaprop-P-ethyl a) mortality and b) fresh-weight dose-response curves for *A. myosuroides* populations following selection for a single generation at  $27.2 \text{ g ha}^{-1}$  fenoxaprop-P-ethyl and a generation with no selection for herbicide resistance after selection at a dose of  $27.2 \text{ g ha}^{-1}$  fenoxaprop-P-ethyl. Lines are predicted values of the dose-response models, symbols are mean values at each dose tested.

### 3.3.5 Differences in plant weight following fenoxaprop-P-ethyl selection

#### i) *Reduction in biomass in the absence of herbicide*

In a number of the fresh weight dose responses fresh weight in the absence of herbicide was significantly reduced compared with the original population, as noted above. To explore this effect, the ‘fresh weight at dose 0’ parameter,  $d$ , was compared with the  $ED_{50}$ , parameter  $e$  (fig. 3.7). A simple correlation test using Pearson’s product moment coefficient indicated a significant, strong negative correlation between fresh weight in the absence of herbicides and  $ED_{50}$  (Pearson’s  $r = -0.92$ ,  $T = 5.12$ ,  $P < 0.01$ ), suggesting a potential trade-off between these two variables. However, this test did not incorporate the large error associated with the model parameters, and alternative experimentation and analysis would be required to test this relationship beyond the simple visualisation here. Fitness trade-offs are considered further in chapter 4.

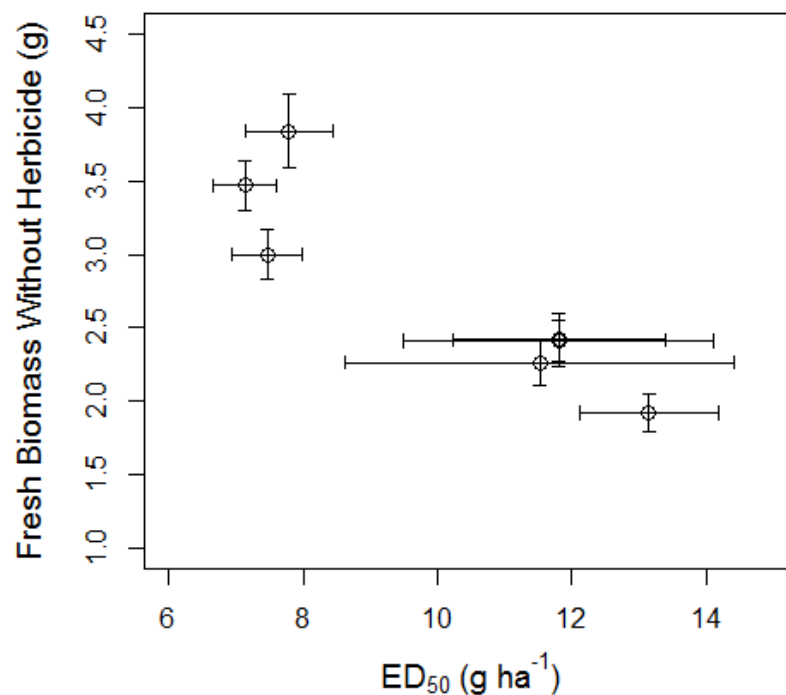


Figure 3.7 Relationship between fresh biomass of plants in the absence of herbicide and  $ED_{50}$  parameter for dose-response curve. Datapoints represent separate populations (including original population and all lines after a single generation of selection), error bars are standard error of parameter estimates.

ii) *Frequency distributions of individual plant weights*

Difference in individual plant responses to a single herbicide dose may provide further insight into the selection dynamics not revealed by dose-responses. For example, as noted in section 3.3.3 above, recurrent selection at  $d=27.2$  appears to have increased plant tolerance to lower herbicide doses, but not significantly increased the overall  $ED_{50}$  of the dose-response. To explore these effects, a frequency distribution was constructed to compare individual plant weights for the original population,  $n=200$ ,  $d=27.2$ ; and  $n=200$ ,  $d=27.2$ ,  $27.2$  following application of  $13.6 \text{ g ha}^{-1}$  fenoxaprop-P-ethyl (fig. 3.8). At  $13.6 \text{ g ha}^{-1}$  most plants from the original population show very low biomass, weighing between 0 and 0.5g (indicating no growth following herbicide application), with a small proportion of individuals of greater biomass. In the  $n=200$ ,  $d=27.2$  population, the frequency distribution is shifted towards greater weights, with the majority of plants now heavier than 0.5g following herbicide application, indicating an increased ability to grow after receiving this dose. For the population  $n=200$ ,  $d=27.2$ ,  $27.2$ , the modal weight category is now 1 to 1.5g, a further increase in growth following herbicide application. This change in distribution as a result of selection can be considered as an alternative model for the underlying trait distribution in figure 3.1. Rather than phenotypic resistance showing variation in maximum survivable herbicide trait, the variation may be considered as a range of phenotypic responses to a single herbicide dose. Applying herbicide therefore selects within this pre-existing variation to shift the trait distribution towards increased biomass (and inferred fitness) at this dose. Potential implications of these dynamics are considered in the discussion. This study was designed to maximise the range of doses tested, to give accurate dose-responses using the resources and time available. Consequently, the data at each individual dose are insufficient to employ a distribution modelling approach, but the dynamics apparent in figure 3.8 suggest the potential for further work exploring individual plant fitness following herbicide application.

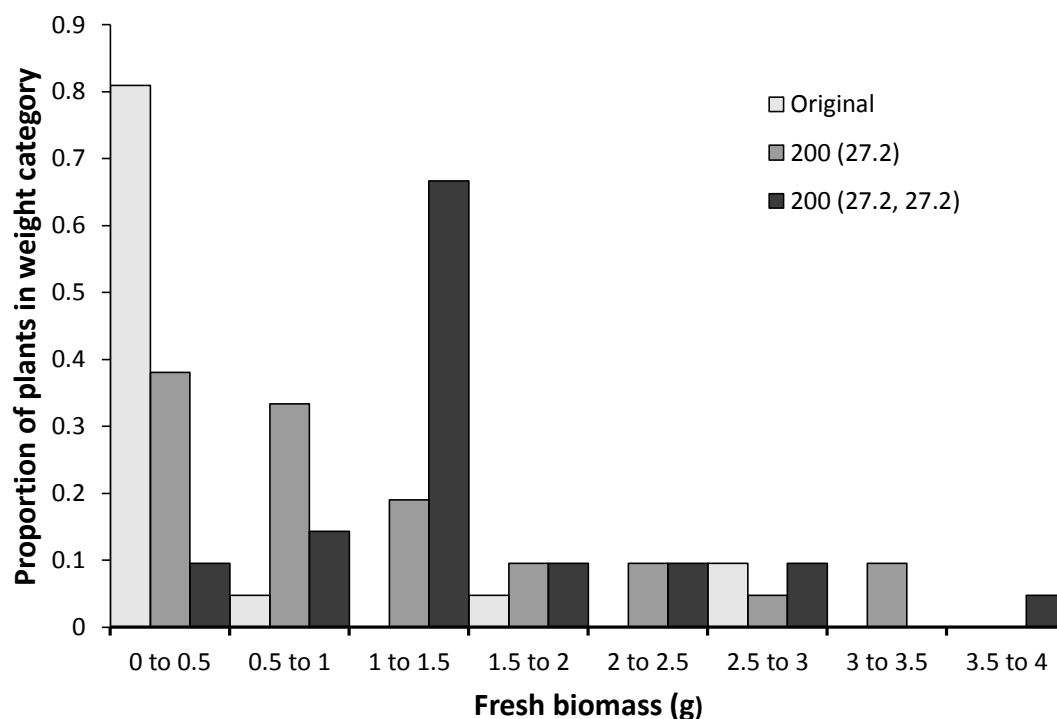


Figure 3.8 Frequency histogram showing the distribution of individual plant weights 21 days after application of  $13.6 \text{ g ha}^{-1}$  fenoxaprop-P-ethyl for the original population, the line selected from 200 individuals at  $27.2 \text{ g ha}^{-1}$  and the line selected a second time under these conditions.

### 3.4 Discussion

#### 3.4.1 Selection within pre-existing variation in low dose herbicide susceptibility can result in increased resistance

Continuous variation in response to low doses of the herbicide fenoxaprop-P-ethyl for a susceptible black-grass population was shown in chapter 2, and is confirmed here in repeated dose-responses (fig. 3.3). In this chapter it is clearly demonstrated that this variation is heritable, and selection within this pre-existing range of variation has significantly increased resistance (fig. 3.4, tables 3.2 and 3.3). Several studies have found similar effects in *Lolium rigidum* (described in the introduction), but this is the first study to demonstrate these effects in *A. myosuroides*, despite its importance as an agricultural pest prone to evolving herbicide resistance. This result also highlights weedy plants as an important example of rapid evolutionary

responses to anthropogenic environmental change (Carroll et al., 2011; Vigueira et al., 2013).

This selection experiment was performed in controlled glasshouse conditions, but the dynamics tested here may also arise in the field. Although the herbicide doses used for selection in this experiment were below recommended rates, it has been suggested that sub-optimal herbicide doses may be received by plants in field conditions as a result of shading by other plants (including the crop), differences in weed germination timing, or mechanical or environmental factors affecting herbicide application (Manalil et al., 2011). Agricultural fields may contain tens of thousands of individual weeds (Cousens and Mortimer, 1995), and so even a small proportion of plants receiving sub-optimal herbicide doses will result in numbers of individuals exposed to selection similar to this study. Selected plants were bulk-crossed at high densities, among plants of the same-age, ensuring outcrossing among selected plants. In the field, susceptible plants may also be present if they escape any herbicide exposure (through the effects described above), and can then outcross with the selected plants and reduce the frequency of resistance conferring alleles (Gressel, 2009). However, if there are spatial differences in weed genotypes and/or consistency of herbicide application, there may be aggregation of resistance genes in smaller patches within a field (Renton, 2013), potentially reflecting the bulk-crossing conditions within this experiment.

### **3.4.2 Impact of herbicide dose and population size in a single generation of selection for herbicide resistance**

Within only 200 *A. myosuroides* individuals sufficient heritable variation in herbicide response was present to select for resistance as typical quantitative trait, with a higher dose establishing a larger selection differential and resulting in a greater increase in resistance. This pronounced quantitative increase in survival was not observed for selection from 100 individuals, possibly suggesting that within the smaller population size there was insufficient genetic variation, which limited the selective response (Willi et al., 2006). Selection from 600 individuals also increased resistance, as might be expected, but did not show further increases beyond those observed for 200 individuals. This may indicate that the larger population subsamples of 600 individuals did not significantly increase the amount of additive

genetic variation able to recombine and increase resistance (following a single generation of selection) compared to 200 individuals. Despite expecting greater genetic variation available for selection in larger population sizes, selection at a dose of 27.2 g ha<sup>-1</sup> fenoxaprop-P-ethyl resulted in a greater increase in LD<sub>50</sub> when selected from 200 individuals than 600 individuals. It may be that in the 600 individual selection line alternative dose-specific fitness mechanisms were selected for that do not contribute to resistance across a range of doses, as measured by LD<sub>50</sub> (discussed further in section 3.4.3 below).

The responses to selection observed in this study also incorporate a number of important stochastic elements. The populations exposed to selection each represent a random sample of the total diversity of the initial susceptible population. Within the bulk-crosses, individual parent combinations may then have important consequences on the resistance profile of their progeny. Although similar dynamics may also occur in the field (for example, through unpredictable variation in herbicide application or pollen movement), an expanded study would be required to fully describe selection for resistance and the effects of population size and selection intensity within a standard quantitative genetic framework.

### **3.4.3 Recurrent selection**

Genetic variation, and subsequently the potential selection differential and heritability, may be expected to decline when a trait is under selection as the pre-existing diversity is exhausted and optimum alleles reach fixation, unless mutation continues to generate further variation (Barton and Keightley, 2002; Hill, 2010). There is ongoing debate regarding the potential limits to directional selection in real populations (Kingsolver and Diamond, 2011), and studies have shown large, long-term responses to directional selection (Moose et al., 2004). In this experiment, recurrent selection did not further significantly increase resistance, and it may be that after a single generation of selection genetic variation has declined such that further quantitative increases in resistance are not possible. Comparison with *L. rigidum* studies suggest the limits of heritability may depend upon the specific herbicide used, with glyphosate showing negligible increases by the third or fourth generation of selection (Busi and Powles, 2009), but significant increases still occurring at the third generation of diclofop-methyl selection (Neve and Powles, 2005b). Given the

uncertainty in  $ED_{50}$  and  $LD_{50}$  values here, it may be that selection is still driving an increased resistance profile, but cannot be detected yet at these levels of replication. Further selection with these experimental populations suggests remaining potential for increased resistance (Neve and Lynch, data not shown).

Recurrent selection may also have selected for increased plant fitness following application of the specific herbicide dose received, rather than increasing survival at higher doses (figure 3.8). If there is dose-specific resistance, as has been demonstrated in *C. reinhardtii* (Lagator, 2012), there may be an optimum dose at which an individual plant is of maximum relative fitness. Expanding the conceptual model described in the introduction, the selection differential will incorporate differences in relative fitness between individuals within the selective environment (Kingsolver and Pfennig, 2007), and so will select for maximum fitness at the applied dose, irrespective of fitness at higher doses (until the population is exposed to them). These dynamics may not be detected by dose-response analyses. Despite previous work exploring the difference between low-dose specific ‘tolerance’ compared to ‘resistance’ which operates at all doses, these processes are rarely considered in an eco-evolutionary context (Baucom, 2009). More broadly, plant fitness is rarely considered following herbicide application beyond survival or weight (Neve et al., 2014). Analyses comparing individual plants fitness across their full life-cycle following herbicide application may provide a framework to study these effects in the future.

#### **3.4.4 Mechanistic basis for observed effects**

While a single gene, target-site resistance mechanism could also be responsible for selection for increased resistance, there was no evidence to suggest this. Rare target-site mutations would not be expected to occur within the numbers of plants selected from in this experiment (discussed in chapter 2). Should any major effect single gene resistance mechanisms have existed then some survival at high doses might have been expected (not observed in fig. 3.3). Finally, any single gene resistance mechanisms would be expected to have been very rapidly selected, resulting in uniform resistance profiles among all individuals following selection, but this was not observed in any experimental line. Instead, the results support a quantitative genetic model of a large number of genes of individually small contribution to



herbicide resistance. The exact nature of many non-target-site resistance mechanisms is as yet unknown, but they are an active area of research (Gaines et al., 2014). The statistically-based quantitative genetic models as described here remain useful however, as genomic approaches have confirmed the assumptions behind these models in several systems, and fully identifying the many genes responsible for quantitative traits is likely to remain a significant challenge (Hill, 2012). Further experimentation is required to establish whether quantitative increases in resistance can be explained by a simple additive model, or if there are potentially more complex synergistic effects between the genes involved.

An alternative possibility for the increase in resistance observed is a resistance mechanism with epigenetic inheritance. The potential for environment to directly change an organism's gene expression and cause heritable changes has not previously been considered in herbicide resistance, but is potentially very important (Neve et al., 2014). The principle of selecting for resistance from a pre-existing phenotypic trait distribution fundamentally remains, but whether this initial variation results from mechanisms inherited epigenetically or according to a standard genetic model will have important impacts on the heritability and ultimate evolution of the trait (Jablonka and Raz, 2009). Quantitative resistance may even prove to be a combination of epigenetic and 'standard' genetic mechanisms, with complex implications for selection.

## **4. Seed production in response to competition for *Alopecurus myosuroides* selected for resistance to fenoxaprop-P-ethyl**

### **4.1 Introduction**

#### **4.1.1 Fitness costs and competition**

Adaptation to a novel stress, such as resistance to a xenobiotic, is often expected to confer a cost in the absence of this stress (Purrington and Bergelson, 1996; Vila-Aiub et al., 2011). Quantifying the cost of resistance is essential to predict the eco-evolutionary dynamics of resistance, from the initial frequencies of resistant plants in a naïve population to its spread among a population during selection, and to what extent a resistant population will impact on crop yields. Fitness costs associated with herbicide resistance will also result in selection against resistance in the absence of herbicide, and may subsequently present opportunities for controlling resistance in weed management.

Herbicide resistance mechanisms, by definition, result in plant phenotypes with increased fitness in the presence of herbicide. The resistance mechanism may also, however, have effects on further phenotypic traits, and potentially overall fitness. A single allele having effects on multiple, seemingly unrelated phenotypic traits is defined as pleiotropy (Stearns, 2010). Pleiotropic costs associated with resistance might be expected for several reasons (Purrington, 2000). If the mutation or mutations that confer resistance compromise normal enzyme function, by impeding substrate binding or altering enzyme kinetics, a fitness cost will arise. For example, a mutation replacing proline 197 with arginine providing target-site resistance (TSR) in the enzyme acetolactate synthase (ALS) also reduced binding of the normal substrate and increased feedback inhibition (Yu et al., 2010). In non-target-site-resistance (NTSR) the same effects may occur, but fitness-costs arising from altered enzyme kinetics of non-target-site resistant isoforms have not yet been demonstrated.

A second category of fitness costs can arise due to a trade-off in resource allocation (Vila-Aiub et al., 2009a). A resistant plant may need to expend energy and resources on its resistance mechanisms that would normally go towards growth and reproduction (Purrington and Bergelson, 1996). It has been suggested that such a fitness cost may be expected for glyphosate resistant *Amaranthus palmeri*, where

resistance is conferred by gene amplification of the herbicide target, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Gaines et al., 2010). There is currently mixed evidence for this, with *Amaranthus palmeri* displaying no fitness costs with up to 75 fold (Vila-Aiub et al., 2014) or 100 fold (Giacomini et al., 2013) amplification of EPSPS; but evidence that EPSPS copy number does confer a negative fitness trade-off in *Amaranthus tuberculatus* (H. Cockerton, PhD thesis).

Fitness costs may also result from changes to life history characters. For example, resistance may be associated with altered germination timing (Délye et al., 2013c). In the absence of herbicide application this delayed germination may result in plants facing stronger competition with earlier established crops or susceptible plants which emerged earlier, as well as potentially reduced growing periods (Purrington, 2000). Finally, fitness costs may arise from altered ecological interactions. If resistance leads to an increased susceptibility to pests or diseases, or plants becoming less attractive to pollinators then fitness will be compromised (Purrington, 2000). The large range of potential effects can make specific ecological costs difficult to study, but have been demonstrated in *Amaranthus hybridus*, where triazine resistant plants were more susceptible to herbivory (Gassmann, 2005).

The expression and extent of fitness costs will depend upon genotype-environment interactions (Raymond et al., 2007). There is mixed evidence for the impact of resource limitation in modifying fitness costs (Purrington, 2000). Resource limitation may also enhance competition between genotypes, and magnify fitness costs, through the differential ability of resistant and susceptible plants to compete for resources. Such changes in fitness costs under different forms of competition have been suggested as a means of limiting the spread of genetically modified crops. An example of this is a transgenic herbicide resistant dwarf tobacco designed to have greater fecundity than wild type when planted in monoculture, but growing very poorly when in competition against wild type tobacco (Al-Ahmad et al., 2005). For weed populations it is also important to measure interspecific competition between weed and crop, as this reflects the conditions experienced by the weed in the field. For example, cytochrome P450 resistant *Lolium rigidum* exhibited reduced competitive ability growing with wheat compared to the susceptible biotype (Vila-Aiub et al., 2009b).

Many studies have sought to quantify fitness costs (reviewed in Vila-Aiub et al., 2009a), highlighting a large range of possible outcomes, dependent upon the specific resistance mechanism. While many cases of herbicide resistance do carry a fitness costs (62% of studies reviewed in (Purrington and Bergelson, 1996), some appear to confer no costs (Menchari et al., 2008) and one case has reported a fitness benefit of resistance even in the absence of herbicide application (Wang et al., 2010). Focussing on target-site based ACCase resistance in *Alopecurus myosuroides*, the fitness costs appear to depend upon the resistance conferring mutations. Two target-site mutations (I1781L and I2041N) incurred no fitness costs, while a D2078G mutation in the same gene resulted in reduced biomass, height and seed production (Menchari et al., 2008). Germination dynamics have also been shown to be affected, with plants carrying the I2041N mutation showing faster germination, and plants with I1781L delayed germination (Délye et al., 2013c). Many of the mechanisms of non-target-site resistance in *A. myosuroides* are still being resolved, and NTSR fitness studies in the species have not previously been performed. However, *Lolium rigidum* plants with ACCase resistance conferred by cytochrome P450s, a large protein family implicated in NTSR (Yuan et al., 2007; Délye, 2013) have been shown to have reduced growth compared to susceptible plants (Vila-Aiub et al., 2005, 2009b).

#### **4.1.2 Impacts of fitness costs**

Much of the initial work addressing resistance fitness costs sought to explain the presence of polymorphisms in resistance traits (Purrington, 2000). An evolutionary equilibrium can be reached maintaining both resistant and susceptible alleles according to the relative strengths of selection for or against resistance alleles in the presence and absence of herbicides (Antonovics and Thrall, 1994; Coustau et al., 2000). Alternatively, if the selection is strong enough that resistant alleles become fixed in a population, the balance of fitness trade-offs may determine the rate at which fixation occurs. Field populations of *A. myosuroides* with ACCase resistance have been shown to contain both resistant and susceptible individuals (Délye et al., 2010b), indicating that there is an evolutionary equilibrium which maintains a proportion of susceptible plants, or that resistance has not yet reached fixation. Resistance-conferring mutations will be expected to arise even before selection is

imposed, and their persistence in a population will be governed by the strength of any trade-offs they impose, given the lack of selection.

An understanding of how fitness costs can impact the evolutionary and ecological dynamics of weed populations may be used to manage agricultural systems in order to delay resistance and minimise impacts on crop yield. In multiple resistant *Lolium rigidum* burying seeds below 8cm was shown to reduce the emergence of target-site ACCase resistant plants compared to the susceptible phenotype (Vila-Aiub et al., 2005), so a ploughing strategy to ensure deep burial could be used to minimise the emergence of resistant plants. Different competitive ability between resistant and susceptible plants could be employed to develop strategies which maintain conditions in which susceptible plants can still compete sufficiently to grow and reproduce, and thus limit the spread of resistance (Gressel, 2009). Alternatively, rotating between different herbicides might delay the evolution of resistance if fitness costs associated with resistance to individual herbicides are large enough to select against specific resistance mutations when exposed to alternative herbicides (discussed in chapters 1 and 5). Simulation models have demonstrated that the optimum strategy to delay resistance depends on the genetic determination of resistance, fitness costs, and life-history traits of the weed in question (Roux et al., 2008). The relative ability of resistant or susceptible weeds to impact the crop may also determine optimum management in order to accurately anticipate the economic impacts as resistance spreads throughout a weed population.

#### **4.1.3 Measurement of fitness and competitive ability**

There are many potential measures that can be used to define fitness, but ultimately they must reflect the ability of an organism to survive at all stages of the life-cycle and successfully reproduce (Orr, 2009). Reproductive output is a commonly used and useful measure of fitness in plants, as it is a result of all stages of the life-cycle up to reproductive maturity and gives a comparison in reproductive output between individuals. However, it must be acknowledged that seed output alone can omit important features, such as dispersal, germination, and longevity of the seeds, and unless carefully controlled will only reliably estimate maternal impacts in outcrossing species, thus omitting potential fitness costs in pollen production and viability (Vila-Aiub et al., 2009a).

When appropriate life history traits for fitness determination are established, there are then several ways of testing its response to competition (Park et al., 2003). The simplest scenario is intraspecific competition, establishing how the response being measured varies per plant at increasing densities of the same plant or the same phenotype (Watkinson, 1980). To test interspecific competition between two groups there are three major categories of experimental design: replacement series, in which plants are grown at a fixed density but with different proportions of the two competitors; simple additive designs where competition is treated as a factor either present or absent; and fully additive designs where both the density and ratios of the two competitors are varied (Freckleton and Watkinson, 2000). A response surface design is the most rigorous form of fully additive competition experiment, and can give measures of competitive effects and plant responses across the range of densities tested (Cousens, 1991; Inouye, 2001).

Attempts to measure fitness costs and competitive ability are frequently confounded by not controlling for the genetic background of plants in which the resistance genes are being studied. This makes it impossible to identify if fitness effects arise from costs associated with resistance alleles, or are due to differences at linked loci or population effects such as non-random mating and inbreeding depression (Vila-Aiub et al., 2011). Alternatively, resistance can be considered at a population level, with all differences between resistant and susceptible phenotypes being measured. While this population based approach cannot assign pleiotropic costs to individual resistance mechanisms, population-level effects which may reflect important field dynamics are incorporated. For example, selection for herbicide resistance may be associated with additional life-history changes not directly caused by the resistance mechanisms (Gundel et al., 2008; Owen et al., 2011).

This study tests whether *Alopecurus myosuroides* in the early stages of selection for resistance to the ACCase herbicide fenoxaprop-P-ethyl expresses a fitness cost in the absence of herbicide compared to the susceptible population from which it was selected. The 'resistant' population is composed of the progeny of a bulk cross following selection for herbicide resistance. As such, it cannot be used to identify costs of specific resistance mechanisms, but emulates the population level dynamics under which herbicide resistance might arise. This study aims to measure the relative competitive ability of the resistant and susceptible phenotypes as measured in

competition with (i) each other and (ii) against wheat (*Tritium aestivum*). In competing against wheat, this study also tests (iii) whether resistant and susceptible plants have different impacts on the growth of wheat.

## **4.2 Materials and methods**

### **4.2.1 Plant material**

The *A. myosuroides* population ‘Oxon’ (see chapter 2), obtained from Herbiseed ([www.herbiseed.com](http://www.herbiseed.com)) was the susceptible population (S) for this experiment. The ‘resistant’ population (R) is derived from Oxon following two generations of selection for survival at 27.2 g ha<sup>-1</sup> fenoxaprop-P-ethyl (see chapter 3).

### **4.2.2 Experimental design**

A response surface design was employed to measure intra- and interspecific competition between the *A. myosuroides* R and S populations and wheat at multiple densities (fig. 4.1). Plants were grown in 15cm diameter pots at 4 different densities, 3, 13, 19 and 37 plants per pot (121, 522, 764 and 1487 plants per m<sup>2</sup> respectively), arranged so that all plants were equally spaced (fig. 4.2). Intra-population competition was measured by growing plants in monoculture at all densities. Inter-population and -specific competition was measured by growing pairwise combinations (S vs R *A. myosuroides*, S *A. myosuroides* vs. wheat and R *A. myosuroides* vs. wheat) in 1:1, 1:2 and 1:3 ratios of the two competing groups across 3, 19 and 37 per plant per pot densities, arranged so that each individual plant had the same number of neighbours of each population/species (illustrated in table 4.1). Not all density/ratio combinations were included (see fig. 1, and table 4.1). For every inter-population and interspecific competition treatment there were 6 replicate pots. For every monoculture treatment there were 9 replicate pots. This resulted in 324 pots overall.

### **4.2.3 Plant growth conditions**

After a dormancy breaking treatment of 3 weeks in the dark at 35°C in dry conditions, *A. myosuroides* seeds of both phenotypes were germinated in petri dishes containing filter paper and 3ml distilled water, maintained in an incubator set to daily photoperiod of 12 hours light at 23°C and 12 hours dark at 9°C. *A. myosuroides*

seedlings of similar size (shoots approx. 3cm) were then transplanted to 15cm diameter pots containing a mixture of 50% 'J Arthur Bowers Top-Soil', 25% 'Levington M2 compost' and 25% sand in October 2012. Wheat seeds (commercial var. KWS Sterling pre-treated with the fungicide prothioconazole) were sown directly at the recommended depth of 4cm (HGCA, 2008). Any *A. myosuroides* plants that did not survive transplanting (recorded two weeks after initial transplant date) were replaced. Plant mortality after this time was attributed to competition. Pots were arranged in 3 blocks each containing 2 replicates of all interspecific competition combinations and 3 replicates of monoculture pots of each plant group at each density. Within blocks pot layout was completely randomised. Pots were maintained in a polythene tunnel approximately 1°C above ambient air temperature. Pots were watered as required. In March 2013 all pots received a fertiliser surface dressing of ammonium nitrate providing the equivalent of 60kg N ha<sup>-1</sup>. Pots received fungicide and insecticide treatments as required. The experiment was harvested in August 2013 when wheat had reached maturity.

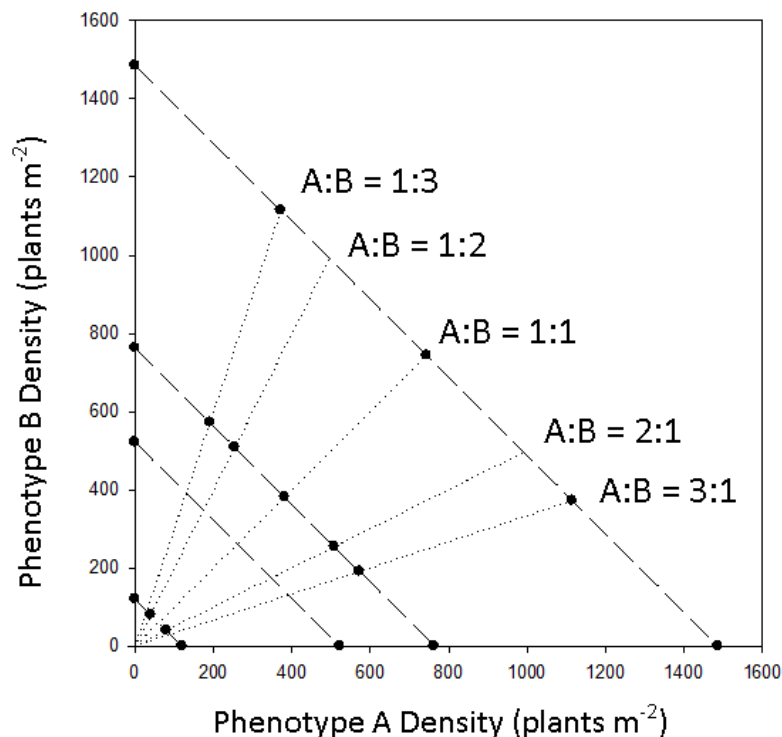


Figure 4.1 Experimental density and competition ratios covered for all pairwise competition combinations. Dashed lines indicate pots with the same total number of plants, intersected by dotted lines showing the ratio of the two competing groups. Solid circles represent experimental treatments included in the design.



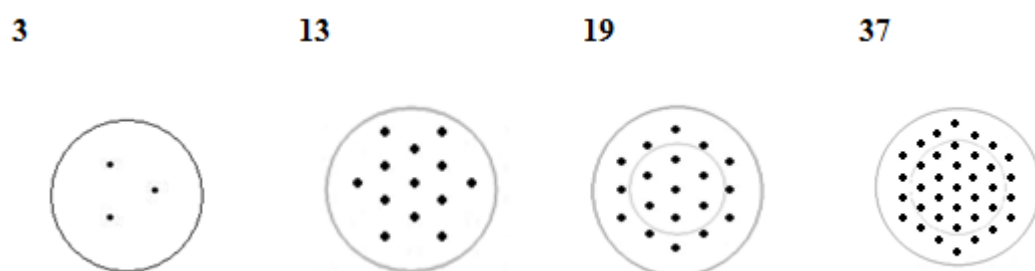
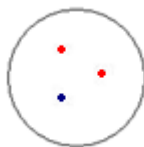
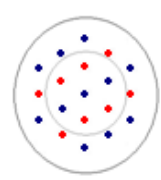
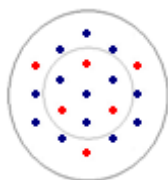
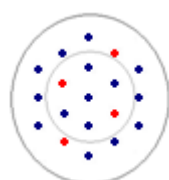
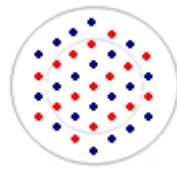
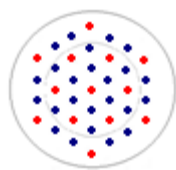
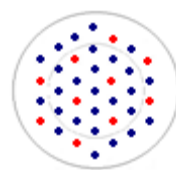


Figure 4.2 Arrangement of plants in 15cm pots at 4 different densities ensuring equal spacing between plants. Plants outside the inner grey ring were not measured.

Table 4.1 Pot layouts of densities and competition ratios tested in semi-factorial response surface design. Different plant populations/species (resistant or susceptible *A. myosuroides* and wheat) tested in pairwise competition; one group is illustrated in blue, the other in red. Plants outside the inner grey ring were not measured. Inter-population –specific competition was not measured at a density of 13 plants per pot as too few plants would be exposed to the correct ratio of neighbours in each replicate pot.

		Ratio of competing groups		
Plants per pot		1:1	1:2 (+2:1)	1:3 (+3:1)
3				
13	No interspecific competition measured			
19				
37				

#### **4.2.4 *A. myosuroides* flower head length to seed output relationship**

When the majority of *A. myosuroides* plants had reached reproductive maturity a sample of 156 flower heads were selected to estimate a flower head length to seed number relationship. As *A. myosuroides* flower heads shed their seeds at maturity (occurring prior to wheat maturity, and hence before harvesting) this relationship was required to derive seed counts from the large numbers of plants measured in the experiment. Monoculture pots were stratified to give equal sample sizes of R and S. Within each R/S strata flower heads were then randomly selected from all densities and across all 3 replicate blocks. After anthesis but before seeds reached complete maturity flower heads were isolated in clear plastic bags to collect seeds during shedding. Bags contained small holes to allow for transpiration and pollen movement. At the end of the experiment seed production from each flower head was counted and head length measured from the point of first spikelet attachment to the tip. The relationship between seed number and flower head length was modelled by a power function (equation 4.1) using non-linear least squares regression in R (R Development Core Team, 2013).

$$\text{Seed number} = a * \text{flower head length}^b \text{ (Eqn. 4.1)}$$

Separate models were fitted for R plants only, S plants only and both populations together. Differences in model fit between the combined model and the two individual models were compared in R using ANOVA (R Development Core Team, 2013).

#### **4.2.5 Competitive responses**

##### *i) A. myosuroides*

To quantify *A. myosuroides* fitness, seed production at the end of the experimental growing period was used as a measure of fecundity. At the point of wheat harvesting, flower head lengths were measured for every *A. myosuroides* plant. Seed production for each *A. myosuroides* head was then estimated from flower head length using the relationship described in section 4.2.4 above. If an individual plant had more than four heads (only common in those grown at the 3 plant per pot density) a random sample of four was measured, to ensure all plants were recorded without delaying wheat harvesting. Seed production for the remaining heads on these plants was

estimated using the mean flower head length for that plant. An estimate of seed output per plant was then obtained by summing seed number per head. This process was repeated for every plant in a pot, giving a mean seed output per plant for every pot. Mean pot values were used for analysis rather than individual plants to avoid pseudo-replication.

#### ii) *Wheat*

Grain yield was the response measured for wheat in this experiment. Wheat spikes were cut from the stem at the base of the lowest spikelet and weighed individually. Plants in the same pot were measured individually but whole pot means were used for analysis to avoid pseudo-replication, as described for *A. myosuroides*.

### 4.2.6 Response surface analysis

Response surface models were fitted to describe how mean per plant responses change with sowing density, and the impacts of competition on this response between all pairwise combinations. For *A. myosuroides*, mean seed output per plant was fitted in the competition model shown below:

$$\log(y_i) = \log\left(\frac{A}{1+B_i(N_i+C_{ij}N_j)}\right) + \varepsilon \text{ (Eqn. 4.2)}$$

where  $y$  is the response measured (here mean seed output estimate per plant) for group  $i$ ,  $A$  the value of this response at 0 density,  $B_i$  the intraspecific competition response of group  $i$ , and  $C_{ij}$  is the competition substitution rate for competing group  $j$  compared to group  $i$ . If  $C$  is equal to 1, then plants of group  $j$  have the same competitive effect on the response of group  $i$  plants as plants of group  $i$ . If  $C$  is greater than 1 group  $j$  plants have an enhanced competitive impact relative to group  $i$ , and vice versa if  $C$  is less than one.  $N_i$  and  $N_j$  are the densities of the two groups  $i$  and  $j$ . Parameter estimates were compared using Z-tests.

The same model (equation 4.2) was used for wheat, with mean total spike weight per plant used as the response.

#### 4.2.7 Replacement series analysis

To consider responses as per unit area measures and compare across the fixed pot densities replacement series analyses were also performed for each pairwise competition combination described above. These were modelled after (De Wit, 1960), described by the function:

$$Y_{ij} = Y_{ii} \cdot \frac{k_{ij} \cdot r_{ij}}{(1-r_{ij})+k_{ij} \cdot r_{ij}} \text{ (Eqn. 4.3)}$$

Where  $Y_{ij}$  is the yield response per unit area of group  $i$  grown with group  $j$  across a range of densities  $r_{ij}$  of group  $i$  relative to group  $j$  for a given overall sowing density (when  $r_{ij}$  is 1 then there is only group  $i$  and when  $r_{ij}$  is 0 there is only group  $j$  and hence the group  $i$  response must be 0).  $Y_{ii}$  is the yield response per unit area of group  $i$  at  $r_{ij} = 1$ . The parameter  $k_{ij}$  is a crowding constant describing the competitive effect across the replacement series. A  $k_{ij}$  value equal to 1 suggests equal competitiveness, as the yield response is directly proportional to sowing frequency. A  $k_{ij}$  value greater than 1 suggests a competitive dominance of group  $i$  over group  $j$ , as replacing group  $i$  plants with group  $j$  plants results in less of a decline in yield than would be expected from the reduced number of group  $i$  plants per unit area. The opposite is true for  $k_{ij}$  values lower than 1. For *A. myosuroides* the yield response was mean seed output per square metre, and for wheat grain yield per square metre. For both measures data were individual pot totals converted to per square metre. Parameter estimates were compared using Z-tests.

## 4.3 Results

### 4.3.1 *A. myosuroides* flower head length to seed output relationship

The power function (equation 4.1) describing seeds per head according to flower head length (in cm) gave parameters  $a = 8.2293 (\pm 1.7815)$  and  $b = 1.3118 (\pm 0.0955)$ , is shown in equation 4.4, illustrated in figure 4.3.

$$\text{Seeds per head} = 8.2993 * \text{flower head length}^{1.3118} \text{ (Eqn. 4.4)}$$

The relationship between seed number and flower head length was also fitted separately for resistant and susceptible plants, but model fits were not significant improvements over the combined model.

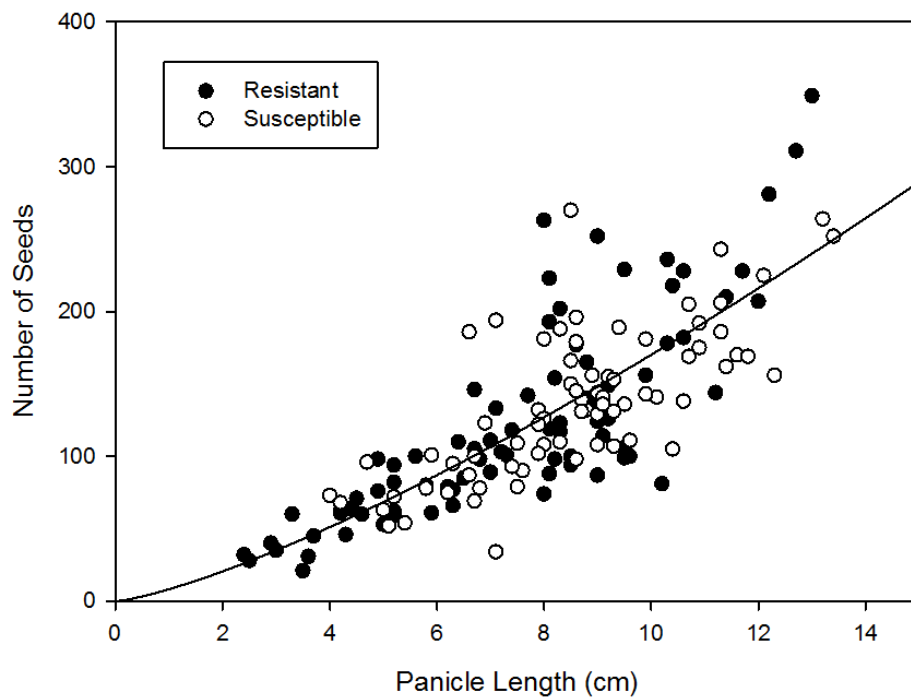


Figure 4.3 Relationship between number of seeds per head and flower head length (cm). Curve represents fitted values for the power function model (eqn. 4.3).

### 4.3.2 Competition between susceptible and resistant *A. myosuroides*

#### i) Response Surface

Estimated seed output for both R and S plants was highly variable at low effective densities ( $N_i + C_{ij}N_j$ ), resulting in poor model fit and high uncertainty in the  $A$  and  $B$  parameters (fig 4.4, table 4.2). There were no significant differences in the parameters for response at 0 density,  $A$  ( $Z = 0.521$ ,  $P = 0.603$ ), or competition response rate,  $B$  ( $Z = 0.555$ ,  $P = 0.579$ ). The trend in the competition substitution rate,  $C$ , suggested that susceptible plants were superior competitors to the resistant population ( $C < 1$  for the effect of R on S, and  $C > 1$  for the effect of S on R), but neither parameter significantly differed from  $C=1$ , the value representing equal competitive ability of the two groups (for R affecting S,  $Z = -0.055$ ,  $P = 0.9558$ ; for S affecting R,  $Z = 0.970$ ,  $P = 0.332$ ).

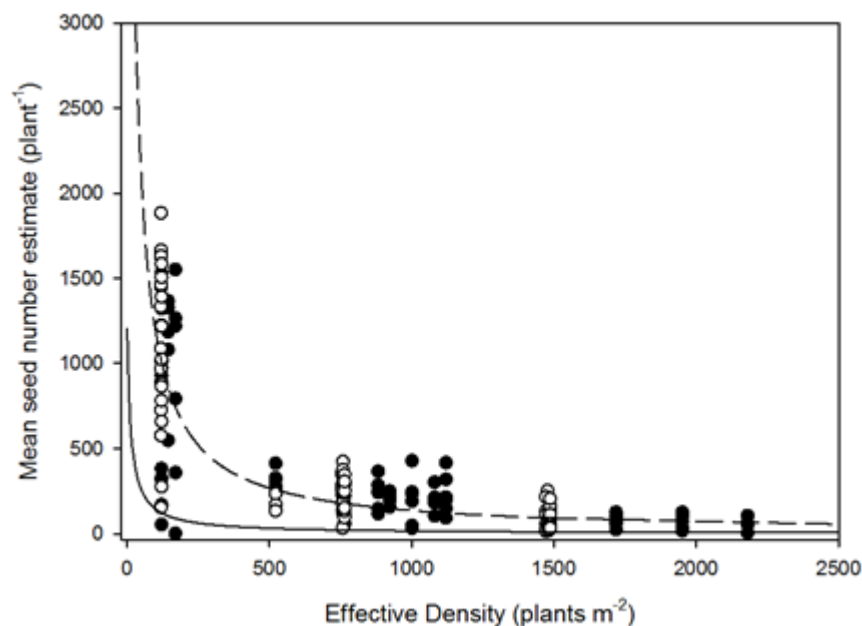


Figure 4.4 Estimated seed output per plant plotted against effective density ( $N_i + C_{ij}N_j$ ) for susceptible (hollow circles and dashed line) and fenoxaprop-P-ethyl resistant (solid circles and line) *A. myosuroides* during inter-population competition. Curves represent fitted values for the competition model (eqn 4.2).

Table 4.2 Summary of regression describing mean seed count (plant<sup>-1</sup>) as a function of plant density for two *A. myosuroides* populations (fenoxaprop-P-ethyl susceptible and resistant) in competition with each other. *A* parameter: seed count at density 0, *B* parameter: competition response rate, *C* parameter: competition substitution rate.

Population <sub>(i)</sub>	Competing with <sub>(j)</sub>	<i>A</i>	<i>B<sub>i</sub></i>	<i>C<sub>ij</sub></i>
Susceptible	Resistant	8625 (±13080)	0.063 (±0.10)	0.99 (±0.03)
Resistant	Susceptible	1206 (±5653)	0.007 (±0.005)	1.62 (±0.64)

ii) *Replacement Series*

As noted for the response surface results, maximum seed output estimates did not differ between phenotypes, and showed a large degree of variability (fig. 4.5, table 4.3). At 121 and 764 plants per m<sup>-2</sup> the crowding constant parameter *k<sub>ij</sub>* did not significantly differ from 1, indicating no difference in competitiveness between the two phenotypes (fig. 4.5 a and b). At total densities of 1487 plants per m<sup>-2</sup> however, susceptible plants were shown to be significantly more competitive than resistant plants in determining resistant seed output (fig 4.5c), with a *k<sub>ij</sub>* parameter of 0.497, significantly less than 1 (*Z*=3.59, *P*<0.001). This effect was not reflected in an increased competitive ability of susceptible plants to produce more seed when in competition with resistant plants however, where the *k<sub>ij</sub>* parameter did not significantly differ from 1 (*Z*=1.53, *P*=0.13).

Table 4.3 Parameter estimates for inter-population *A. myosuroides* replacement series describing mean seed count (seeds m<sup>-1</sup>) as a function of proportion of plant density for two *A. myosuroides* populations (fenoxaprop-P-ethyl susceptible and resistant) in competition with each other. *Y<sub>ii</sub>* parameter: seed count (seeds m<sup>-2</sup>) when only population *i* sown, *k<sub>ij</sub>* parameter: crowding constant describing effect of competition, at *k<sub>ij</sub>*=1 populations are of equal competitiveness.

Population ( <i>i</i> )	Competing with ( <i>j</i> )	Density (plants m <sup>-2</sup> )	<i>Y<sub>ii</sub></i>	<i>k<sub>ij</sub></i>
Susceptible	Resistant	121	128300 ±12900	1.515 ±0.767
		764	227400 ±17460	1.171 ±0.321
		1487	175000 ±27030	0.937 ±0.609
Resistant	Susceptible	121	125600 ±10600	1.018 ±0.392
		764	181800 ±17840	1.371 ±0.511
		1487	192000 ±13270	0.497 ±0.140

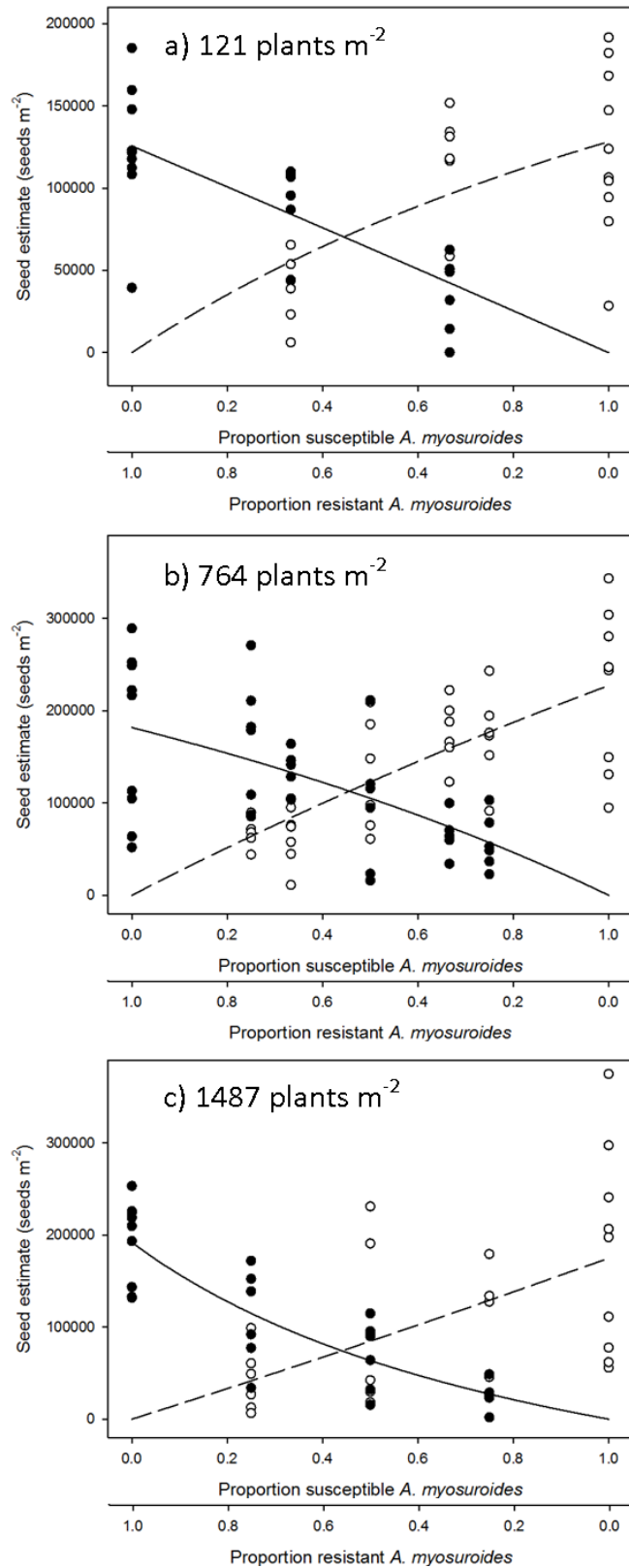


Figure 4.5 Seed estimates (metre<sup>-2</sup>) for susceptible (hollow circles, dashed line) and resistant (solid circles, solid line) *A. myosuroides* in replacement series in competition with each other at 3 different total sowing densities. Datapoints represent individual pot values. Curves represent fitted values for the replacement series models.



### 4.3.3 Susceptible and resistant *A. myosuroides* in competition with wheat

#### i) *Response surface*

##### *A. myosuroides* seed production

As in intraspecific competition between the two *A. myosuroides* populations, responses were highly variable (fig. 4.6, table 4.4). There was no significant difference in maximum seed output (A parameter  $Z = 0.805$ ,  $P = 0.421$ ) or competition response rate parameter  $B$  ( $Z = 0.799$ ,  $P = 0.424$ ) between resistant and susceptible populations when competing with wheat. The susceptible group was more competitive than wheat, with a C value of 0.4, significantly less than 1 ( $Z = -5.15$ ,  $P < 0.001$ ). There was not a significant difference in competitiveness between the resistant population and wheat ( $C=1.11$ ,  $Z = 0.271$ ,  $P = 0.787$ ).

##### *Wheat yield*

The same wheat phenotype was tested against both *A. myosuroides* population, so the A and B parameters, which do not measure the effects of inter-specific competition, do not differ. There was a trend for both *A. myosuroides* phenotypes to outcompete wheat, and susceptible *A. myosuroides* more competitive than resistant *A. myosuroides*, but neither were significantly different from 1, indicating no significant difference in competitiveness between either *A. myosuroides* population and wheat in determining wheat panicle weight (fig. 4.7, S *A. myosuroides*:  $Z = 1.86$ ,  $P = 0.064$ ; R *A. myosuroides*:  $Z = 1.23$ ,  $P = 0.218$ ).

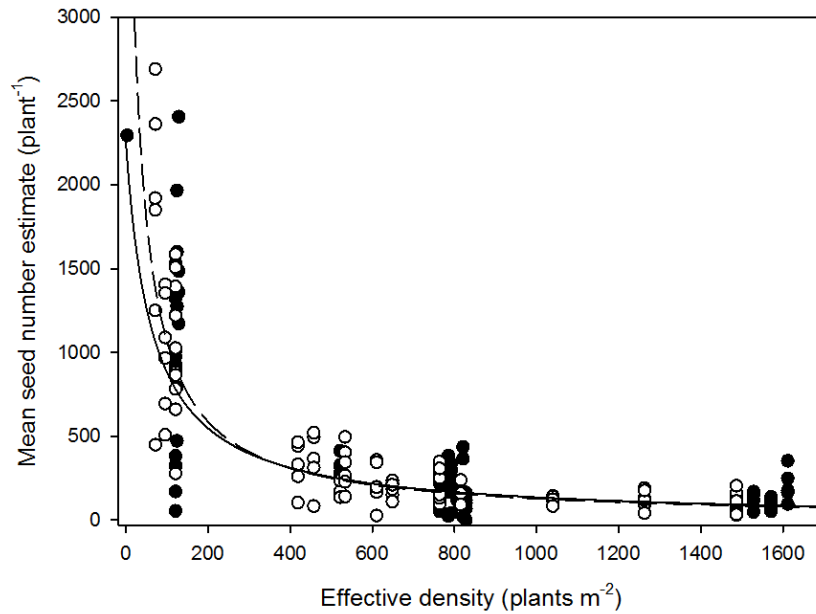


Figure 4.6 Estimated seed output per plant plotted against effective density ( $N_i + C_{ij}N_j$ ) for susceptible (clear circles and dashed line) and fenoxaprop-P-ethyl resistant (solid circles and line) *A. myosuroides* in competition against wheat. Curves represent fitted values for the competition model (eqn 4.2).

Table 4.4 Summary of regression describing mean seed count ( $\text{plant}^{-1}$ ) as a function of plant density for two *A. myosuroides* populations (fenoxaprop-P-ethyl susceptible and resistant) in competition with wheat.  $A$  parameter: response at 0 density,  $B$  parameter: competition response rate,  $C$  parameter: competition substitution rate.

Population <sub>(i)</sub>	Competing with <sub>(j)</sub>	$A$	$B_i$	$C_{ij}$
Susceptible	Wheat	5718 ( $\pm 4112$ )	0.044 ( $\pm 0.033$ )	0.40 ( $\pm 0.12$ )
Resistant	Wheat	2255 ( $\pm 1255$ )	0.016 ( $\pm 0.010$ )	1.11 ( $\pm 0.41$ )

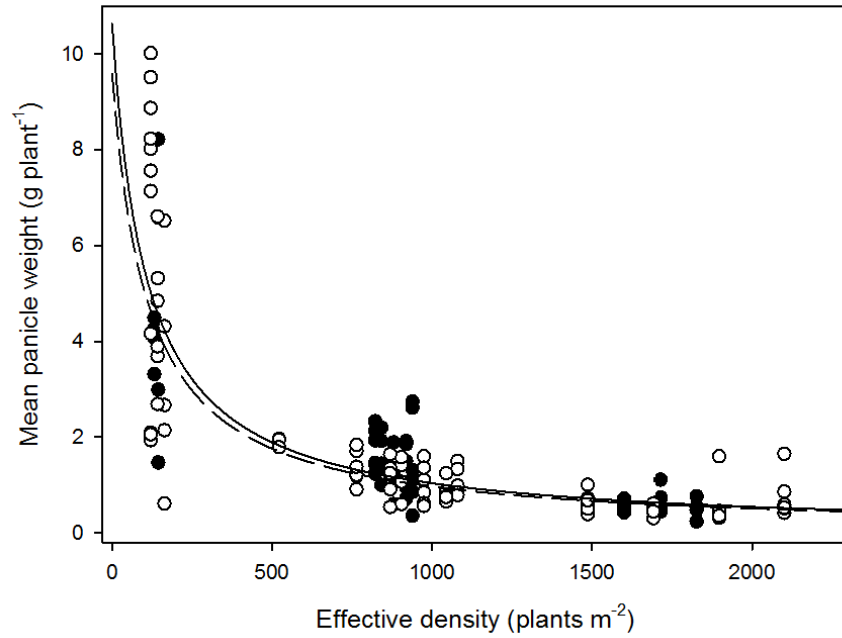


Figure 4.7 Mean total panicle weight per plant plotted against effective density ( $N_i + C_{ij}N_j$ ) for wheat in competition with susceptible (clear circles and dashed line) and fenoxaprop-P-ethyl resistant (solid circles and line) *A. myosuroides*. Curves represent fitted values for the competition model (eqn 4.2).

Table 4.5 Summary of regression describing mean total panicle weight ( $\text{g plant}^{-1}$ ) as a function of plant density for wheat in competition with two *A. myosuroides* populations (fenoxaprop-P-ethyl susceptible and resistant). *A* parameter: response at 0 density, *B* parameter: competition response rate, *C* parameter: competition substitution rate.

Population <sub>(i)</sub>	Competing with <sub>(j)</sub>	<i>A</i>	<i>B<sub>i</sub></i>	<i>C<sub>ij</sub></i>
Wheat	Susceptible	9.59 ( $\pm 2.54$ )	0.0089 ( $\pm 0.003$ )	1.55 ( $\pm 0.30$ )
Wheat	Resistant	10.64 ( $\pm 2.83$ )	0.0093 ( $\pm 0.003$ )	1.30 ( $\pm 0.25$ )

ii) *Replacement series*

*A. myosuroides* seed production

The replacement series models describing the impact of wheat on *A. myosuroides* (table 4.6, fig. 4.8) show a trend for *A. myosuroides* to be more competitive than wheat, especially at the lowest total plant densities (fig. 4.8a), but due to the poor model fit for parameter  $k_{ij}$  did not differ from 1 for either *A. myosuroides* phenotype at any density.

*Wheat yield*

The replacement series models showing the impact of *A. myosuroides* on wheat are shown in figure 4.9 (parameter estimates table 4.6). At the lowest density of 121 total plants  $\text{m}^{-2}$  there is clearly a greater competitive impact of the two *A. myosuroides* phenotypes on wheat compared to intraspecific wheat competition (fig. 4.9a). The relationship was very similar for both *A. myosuroides* phenotypes, and the  $k_{ij}$  parameter for both was significantly less than one (Susceptible:  $Z=8.63$ ,  $P<0.001$ ; Resistant:  $Z=6.36$ ,  $P<0.001$ ). At the intermediate density of 764 total plants  $\text{m}^{-2}$  there is a greater competitive impact of susceptible *A. myosuroides* (fig. 4.9b) with a  $k_{ij}$  value of 0.58, significantly less than 1 ( $Z=3.82$ ,  $P<0.001$ ), while the  $k_{ij}$  value for resistant *A. myosuroides* did not differ from 1, but the difference between this parameter for the two phenotypes was not quite significant ( $Z=1.84$ ,  $P=0.066$ ). At the highest densities of 1487 total plants  $\text{m}^{-2}$  both susceptible and resistant *A. myosuroides* had a  $k_{ij}$  value lower than but not significantly different to 1, and did not differ from each other.

Table 4.6 Parameter estimates for replacement series of resistant and susceptible *A. myosuroides* phenotypes in competition with wheat.  $Y_{ii}$  parameter: seed count/wheat yield (seeds  $\text{m}^{-2}$  or wheat yield  $\text{m}^{-2}$ ) when only population  $i$  sown,  $k_{ij}$  parameter: crowding constant describing effect of competition, at  $k_{ij}=1$  populations are of equal competitiveness.

Biotype ( $i$ )	Competing with ( $j$ )	Density (plants $\text{m}^{-2}$ )	$Y_{ii}$	$k_{ij}$
Susceptible	Wheat	121	119400 $\pm$ 12530	2.34 $\pm$ 1.39
		764	211900 $\pm$ 24160	2.06 $\pm$ 1.01
		1487	184500 $\pm$ 22220	1.80 $\pm$ 1.06
Resistant	Wheat	121	126400 $\pm$ 13370	2.53 $\pm$ 1.70
		764	183400 $\pm$ 20720	1.15 $\pm$ 0.49
		1487	193300 $\pm$ 15100	1.67 $\pm$ 0.62
Wheat	Susceptible	121	959 $\pm$ 56.0	0.31 $\pm$ 0.08
		764	1300 $\pm$ 73.1	0.58 $\pm$ 0.11
		1487	1160 $\pm$ 92.6	0.67 $\pm$ 0.23
Wheat	Resistant	121	957 $\pm$ 60.4	0.30 $\pm$ 0.11
		764	1350 $\pm$ 91.6	1.10 $\pm$ 0.26
		1487	1180 $\pm$ 70.3	0.75 $\pm$ 0.18

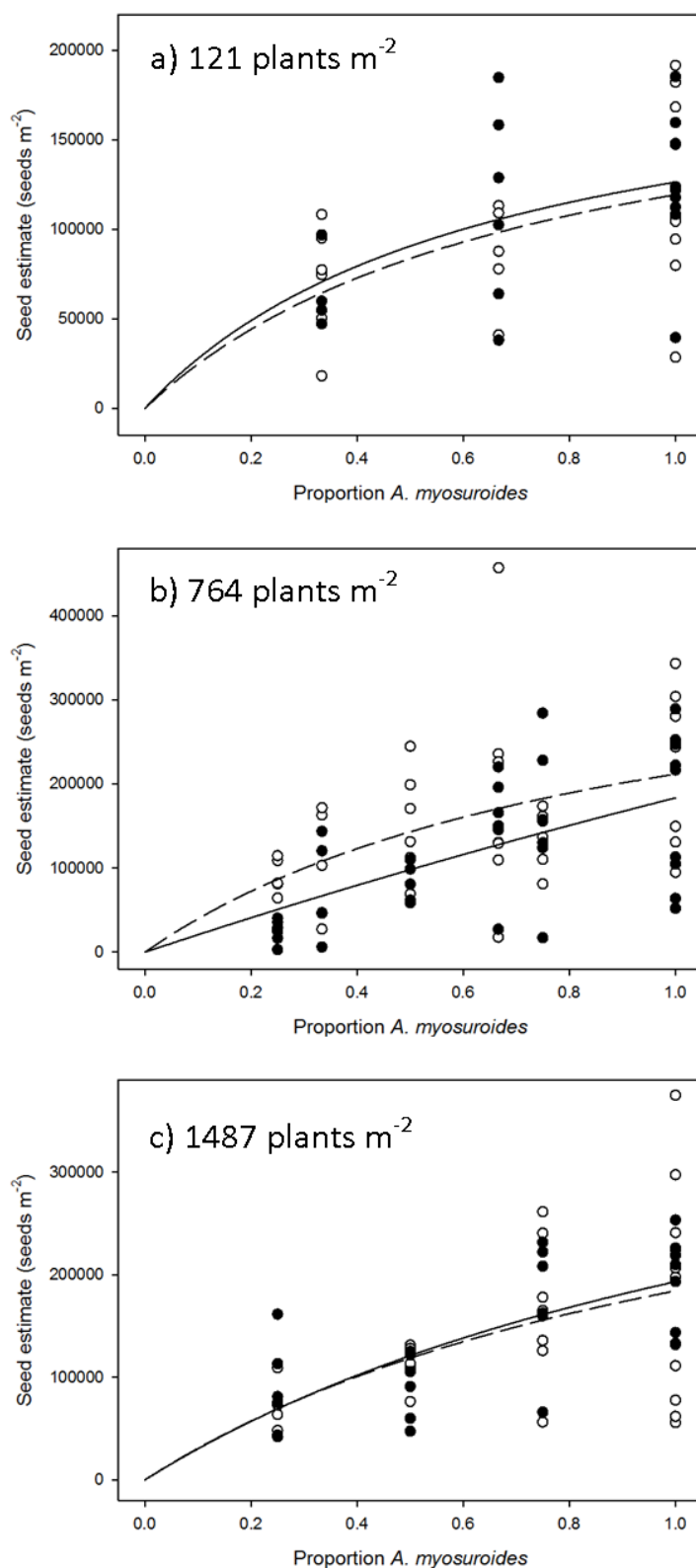


Figure 4.8 Seed estimates ( $\text{metre}^{-2}$ ) for susceptible (hollow circles, dashed lines) and resistant (solid circles, solid line) *A. myosuroides* in replacement series in competition with wheat at 3 different total sowing densities. Datapoints represent individual pot values. Curves represent fitted values for the replacement series models.

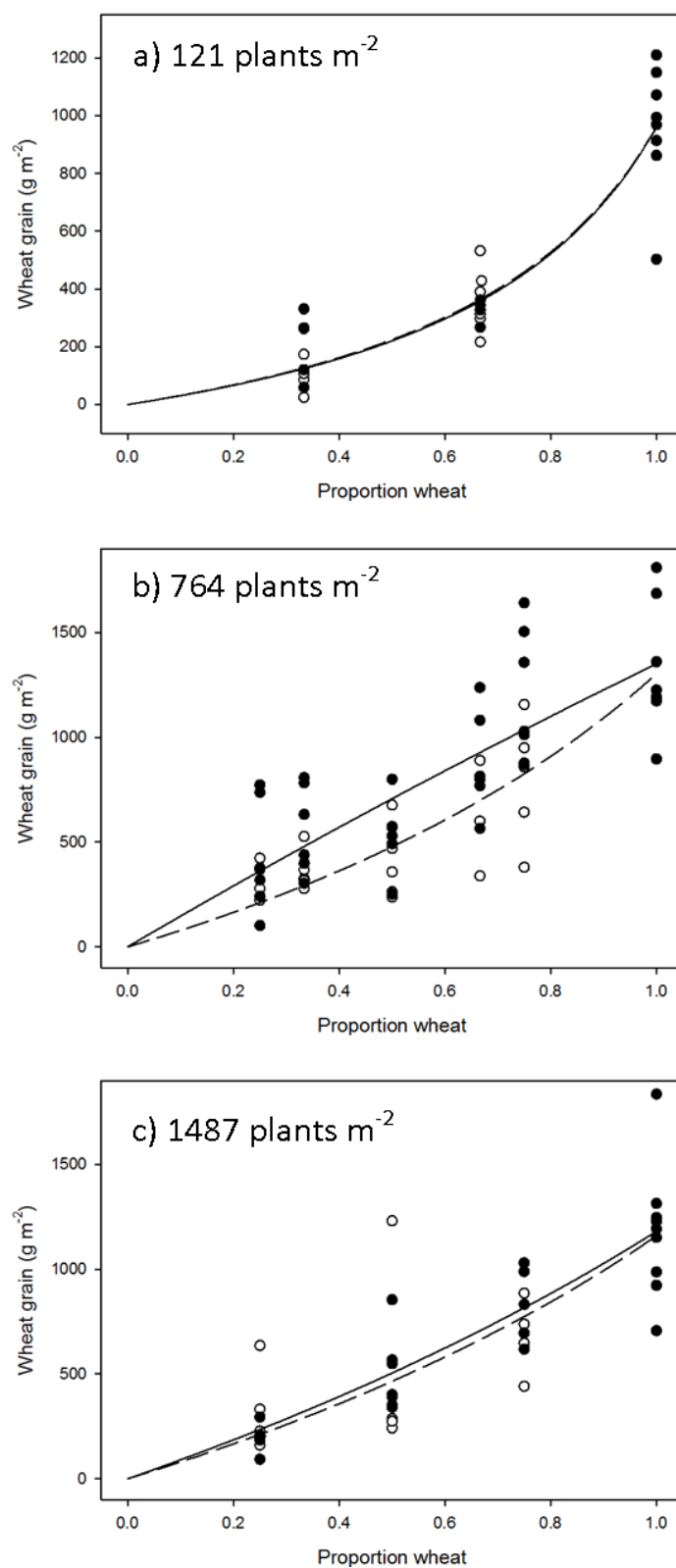


Figure 4.9 Wheat grain yield (metre<sup>-2</sup>) replacement series in competition with susceptible (hollow circles, dashed lines) and resistant (solid circles, solid line) *A. myosuroides* at 3 different total sowing densities. Datapoints represent individual pot values. Curves represent fitted values for the replacement series models.

## 4.4 Discussion

### 4.4.1 Fitness costs associated with selection for resistance

The results of this study suggest that selection for fenoxaprop-P-ethyl resistance in *A. myosuroides* has not conferred a major reduction in growth or competitive ability. Herbicide selection has provided a clear fitness benefit in the presence of herbicide (chapter 3), but no strong costs in its absence, and so resistance could be expected to spread successfully at this stage of its evolution. The overall similarity between resistant and susceptible populations shown here presents no means of exploiting a fitness cost in order to limit resistance evolution, as proposed by Vila-Aiub et al. (2011). There was a significant competitive advantage for the susceptible population over resistant plants at very high weed densities, and it may be that a minor fitness cost conferred by resistance was only detectable where high competition resulted in resource limitation (Purrington, 2000). However, even if this advantage provided an opportunity for resistance management, the very large weed populations required are likely to be impractical and uneconomic as part of a weed management strategy (Diggle and Neve, 2001). Even if feasible within a weed management strategy, high weed densities are unlikely to occur in the early stages of selection for resistance tested here, as most individuals in the seed bank would still be susceptible and killed by normal herbicide rates.

In general, there is mixed evidence regarding the extent of herbicide resistance associated fitness costs (Vila-Aiub et al., 2009a). The mechanisms providing increased resistance for this experimental population are unknown, but are expected to be forms of NTSR (chapter 3). Few studies have previously studied fitness costs as a result of NTSR, but cytochrome P450 enhanced metabolism resistance in *Lolium rigidum* has been demonstrated to negatively impact competitiveness (Vila-Aiub et al., 2009b). The lack of evidence for a fitness trade-off may be a result of resistance selection being at an early stage. The cytochrome P450 example above, in common with most resistance cost studies, used plants derived from agricultural populations with high levels of resistance selected over many years. In contrast, the resistant population in this experiment is a result of only two generations of selection. As suggested in chapter 3, it is likely that further selection will continue to increase resistance, as demonstrated in *L. rigidum* (Neve and Powles, 2005b). Any



costs of resistance may build up quantitatively alongside with the resistance trait, and may only be evident after further selection and at greater levels of resistance. Alternatively, the early stages of selection for resistance as tested here may impose the greatest fitness costs, as there has been the least amount of time for selection to counter these costs. Costs could be reduced over time as compensatory mutations against costs inherent in the resistance mechanisms arise, as demonstrated in antimicrobial resistance (Schulz zur Wiesch et al., 2010); or as linkage disequilibria between the resistance mechanisms and other cost conferring genes are eventually lost.

It should also be recognised that this study examines only a limited component of the *A. myosuroides* reproductive life history. The period of exposure to competition here began when pre-germinated *A. myosuroides* seeds were transplanted at the same growth stage. In practice, any costs in germination, emergence and early growth could drastically affect the relative fitness of resistant and susceptible phenotypes up to this point, as observed for cytochrome P450 based resistance in *L. rigidum* (Vila-Aiub et al., 2005) and for some target-site ACCase mutations in *A. myosuroides* (Délye et al., 2013c). This experiment was only able to measure maternal fitness effects on seed output, as plants of each phenotype were not isolated to prevent pollen movement between them (Vila-Aiub et al., 2011). A paternal fitness cost of resistance may limit pollen production or viability, with the impact on the resistance population dynamics depending upon the population composition (i.e. whether there remain sufficient susceptible plants producing viable pollen) and mode of inheritance of resistance. Using seed output as the final fitness measure also assumes the viability of both resistant and susceptible produced seeds is equal. In an agricultural scenario all of these additional life history changes can have an impact (Colbach et al., 2007).

#### **4.4.2 Competition with wheat**

The study demonstrated that both resistant and susceptible *A. myosuroides* populations were highly competitive growing with wheat, and would be expected to greatly affect crop yields. *A. myosuroides* is recognised as being a highly competitive weed (Moss, 2013b), but the extremely strong impact on wheat growth observed here is perhaps surprising. A similar experiment with *Lolium rigidum* showed that

approximately 10 *L. rigidum* plants were required to reduce wheat yields by the same amount as another wheat plant (Pedersen et al., 2007), compared to a single *A. myosuroides* plant having this great an impact in this study. This may in part reflect an experimental bias in favour of *A. myosuroides*, as *A. myosuroides* was pre-germinated to ensure an equal starting point for all individuals, while wheat was directly sown, emerging at least two weeks later. This highlights the agronomic importance of ensuring sufficient crop density before weeds are established (Olsen et al., 2006, 2012), including *A. myosuroides* (Lutman et al., 2013).

#### **4.4.3 *A. myosuroides* variability**

The *A. myosuroides* responses in this chapter showed very high levels of variability within treatments, resulting in uncertainty in the models and potentially masking differences between the resistant and susceptible populations. There were consistent trends that susceptible *A. myosuroides* was more competitive than the resistant population, but differences were mostly non-significant. Further experimentation may confirm these differences in competitiveness, but the high level of variation observed in *A. myosuroides* in this study would make the necessary levels of replication difficult to achieve within factorial competition experiments. Previous studies have also shown significant variation in *A. myosuroides* seed output (Lutman et al., 2013), driven largely by variability in the numbers of panicles per plant (Dalbiès-Dulout and Doré, 2001; Moss et al., 2010). Alternative measures such as above ground biomass are potentially more consistent (Pedersen et al., 2007), but quantification of seed production is ultimately required to predict whether resistance impacts the reproductive success of plants, and will spread and become problematic.

*A. myosuroides* is genetically diverse within populations, and it has been suggested that the high phenotypic variation in traits such as tiller number is caused by underlying genetic variation (Chauvel and Gasquez, 1994), rather than highly plastic development. If this is the case, then high levels of standing variation might also be expected for other traits in the population tested, including herbicide response. As described in chapters 2.1 and 3.1, pre-existing variation in herbicide response enables rapid selection for resistance. More broadly, high genetic variation has been suggested as a unifying feature in some of the weeds most able to evolve herbicide resistance (Heap, 2014b). Furthermore, as such a high level of variation was

observed in both the resistant and susceptible populations, the two generations of selection for fenoxaprop-P-ethyl resistance do not appear to have reduced variability in seed output, indicating high levels of overall genetic diversity remain, potentially including further variation in herbicide response which might lead to continued increases in resistance.

## 5. Herbicide dose rotation in *Chlamydomonas reinhardtii*

### 5.1 Introduction

In most herbicide-based weed control strategies, herbicide resistance is only considered after it has evolved (Beckie, 2006). It is increasingly being recognised that successful weed management strategies need to proactively consider the evolutionary consequences of their implementation, to limit or delay herbicide resistance (Neve et al., 2009). In order to reduce the evolution of resistance, weed management strategies must minimise continuous exposure to the same herbicide imposed selection pressures (Norsworthy et al., 2012). Non-chemical control may offer a means of diversifying weed management strategies (Harker and O'Donovan, 2013), and cultural control is expected to be included in integrated weed management strategies (Lutman et al., 2013). However, non-chemical control is generally labour-intensive and requires multiple techniques to reach the efficiency of chemical control (Bastiaans et al., 2008). Therefore, strategies must also diversify control, and hence selection pressures, within herbicide based weed management.

Cycling between different herbicide modes of action is a commonly employed means of introducing temporal heterogeneity in order to delay resistance in herbicide-based weed control (Beckie and Reboud, 2009). In a rotation strategy, there is a reduced period of selection for each individual environment, and subsequently there will be a more limited evolutionary response within a given time-frame (Futuyma and Moreno, 1988). If resistance to a specific herbicide is associated with a pleiotropic fitness cost, when not exposed to that herbicide (including in the presence of other herbicides), there will be selection against this resistance allele, delaying resistance evolution even under long-term herbicide exposure (Gressel and Segel, 1990). For these cycling dynamics to limit resistance, it is generally assumed resistance to each herbicide must evolve via independent mechanisms (Neve et al., 2014). As discussed in earlier chapters, applying different doses of the same herbicide may also select for different resistance mechanisms (Neve et al., 2009), and therefore rotating dose strength for a single herbicide may provide an alternative means of retarding resistance in weed control (Gardner et al., 1998).

The theoretical model of dose dependent resistance evolution in weedy plants presented in previous chapters suggested that low doses will select for weaker dose-specific NTSR mechanisms, while higher doses will select for major effect TSR mechanisms (Neve et al., 2014, and discussed further in chapters 2.1 and 3.1). The most extreme dose strength cycling strategies might alternate between receiving a very high dose and not being exposed to herbicide. When not exposed to herbicide, there is no selection for herbicide resistance, and instead any pleiotropic costs associated with resistance alleles will result in selection against resistance (chapter 4). Where a dose rotation strategy is employed, selection for or against each resistance mechanism will depend to what extent, if any, resistance at one dose provides a cost or benefit to resistance in the alternative dose or doses. Mathematical models have been used to demonstrate the potential for dose-rotation in weed management strategies (Gardner et al., 1998). Simulated *Lolium* populations with pre-existing additive genetic variation that could be selected by low herbicide doses (suggested as being provided by NTSR mechanisms), and a rare major effect gene conferring resistance to both high and low herbicide doses (representing a strong TSR mechanism), were compared under dose-rotation, and continuous high or low doses. Dose rotation was shown to control population sizes and delay resistance for longer than either continuous dose, with success dependent on the specific doses in a rotation.

Despite these potential advantages, there may also be risks in introducing temporal dose heterogeneity in weed management. Introducing environmental heterogeneity may select for generalists, which maximise fitness in all environments encountered (Kassen, 2002). In some cases, generalists have been shown to evolve superior fitness than specialists across all environments (Buckling et al., 2007). In herbicide rotation strategies, the generalist evolutionary strategy is to evolve cross-resistance to multiple herbicides (Gressel, 2002). Cross-resistance has been documented for many herbicide resistance mechanisms, and is an important concern for resistance management (Beckie and Tardif, 2012). In dose rotation strategies, high-level resistance that greatly increases fitness at both doses encountered may rapidly evolve and spread across a population if the rotation strategy does not keep major resistance mechanisms at limited frequencies (Gardner et al., 1998). It has also been suggested

that incorporating low doses in a herbicide strategy may risk increasing the rate of resistance evolution (Gressel, 2009, 2011).

Medium and longer term evolutionary ecological and population dynamic processes are difficult to test experimentally in higher plants due to slow generation times and the space required to grow large populations. Microbial experimental evolution can be used to test many of these evolutionary ecology principles but with the convenience of using rapidly replicating microorganisms (Buckling et al., 2009). The unicellular alga *Chlamydomonas reinhardtii* is an excellent model system for herbicide resistance, as it is susceptible to, and can evolve resistance to, a range of commercial herbicides that target higher plants (Reboud et al., 2007). Previous studies on herbicide resistance in *Chlamydomonas reinhardtii* have shown that cycling herbicide modes of action can have diverse effects on resistance evolution (Lagator et al., 2013b); that mixtures of different modes of action can slow resistance if mixed at higher doses, but risks accelerating resistance and evolving cross-resistance at lower doses (Lagator et al., 2013a); and that the highest levels of herbicide resistance can confer the lowest fitness costs in the ancestral (herbicide free) environment (Vogwill et al., 2012).

In this chapter the potential impacts of introducing temporal environmental heterogeneity by rotating doses of a single herbicide are explored. This is tested using experimental evolution of *C. reinhardtii* in response to the long-chain fatty acid synthesis inhibiting herbicide S-metolachlor (HRAC group K3). S-metolachlor was used in this experiment because of the consistency of resistance evolution observed in previous *C. reinhardtii* studies (Lagator, 2012). Continuous application of a single herbicide dose is compared with three different rotation strategies that cycle between higher and lower doses (comparing three different levels of environmental heterogeneity), whilst maintaining the same mean dose across the rotation period. These four strategies are compared for three different mean doses. Within each rotation strategy, two different levels of temporal variation are also tested. This experimental framework is used to test three major hypotheses: (i) Revolving dose strategies can slow adaptation to herbicide compared to exposure to a uniform dose. (ii) The rate of resistance evolution and level of resistance evolved will depend on mean herbicide dose, dose rotation strategy and rotation phase duration. (iii) Resistance will confer a fitness cost in the absence of herbicide, the

strength of which will also depend on mean herbicide dose, dose rotation strategy and rotation phase duration.

## **5.2 Materials and methods**

### **5.2.1 Founding populations**

*Chlamydomonas reinhardtii* wild-type positive mating strain CC-1690 from the *Chlamydomonas* Resource Centre was the original stock population for the experiment. This strain had been cultured in liquid Bold's medium in experimental culture conditions for over 1000 generations before this experiment and was therefore well adapted to laboratory conditions. Eight weeks before the commencement of the experiment, six separate control populations were established by transferring approximately 125000 cells from the stock population to individual culture tubes (as described below in section 5.2.2). These six independent populations were used to found the six technical replicates of each experimental regime (section 5.2.5 below), as well as being maintained as controls under standard culture conditions without herbicide for the duration of the experiment.

### **5.2.2 Culture conditions**

Modified Bold's Medium (subsequently 'BM', (Harris, 2009) was the culture medium for all conditions. Populations were cultured in 25 x 150 mm disposable borosilicate glass culture tubes. The total BM plus herbicide volume for all experimental conditions was 20ml. Populations were maintained in an orbital shaker incubator at 28°C and 180 rpm under continuous light exposure provided by six fluorescent tubes located in the incubator lid (Osram L30 W/21-841 cool white bulbs providing a light intensity of 161  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ). Every seven days a volume of culture medium containing approximately 125000 cells (estimated from absorbance at 750 nm, see section 5.2.3 below) was transferred into fresh medium, seven days being the period required for the ancestral population to reach stationary phase in the absence of herbicide (population size at stationary phase approximately  $3.1 \times 10^7$  cells). Initial population size was standardised in this way to allow weekly population growth comparisons and to prevent evolving populations from going extinct. A transfer volume limit was imposed to minimise transfer or dilution of herbicide between culture tubes (section 5.2.5 below). If the estimated volume of

culture medium required to transfer 125000 cells was above 200µl (1% of the total media volume), only 200µl was transferred, and the cell count deficit was made up using the relevant control stock population.

### 5.2.3 Cell count estimates

Cell count estimates were determined using a calibration curve of OD<sub>750</sub> of the culture (optical density at 750 nm, measured using a Jenway 6315 bench-top spectrophotometer) versus cell number per ml of culture. To produce the calibration curve a sample of the stock population was cultured for 7 days and then diluted in a concentration series. The OD<sub>750</sub> of each solution in the serial dilution was measured. From each solution a 10µl sample was diluted with 985 µl of distilled water in a microcentrifuge tube. To this, 5 µl Lugol's solution (1g iodine, 0.5g potassium iodide in 100ml distilled water) was added, and the mixture was gently shaken. 10µl of this mixture was loaded onto a haemocytometer (Neubauer, 1/400mm<sup>2</sup>, 0.11mm depth) and a cover glass was placed on top. The haemocytometer was placed under a light microscope, and cell number within the counting chamber was recorded. Cell counts were multiplied by 10<sup>4</sup> to give cell density in cells ml<sup>-1</sup>, as the counting chambers hold 0.1µl of sample. Serial dilutions were repeated for three more populations cultured from the stock population. Regression analysis of OD<sub>750</sub> versus cell density was then performed (Fig. 5.1) to derive a quadratic function (after Lagator, 2012, PhD thesis) describing the relationship as:

$$\text{Cell density} = 1982383 (\pm 132155) * OD_{750} + 200794 (\pm 121233) * OD_{750}^2$$

(Eqn. 5.1)



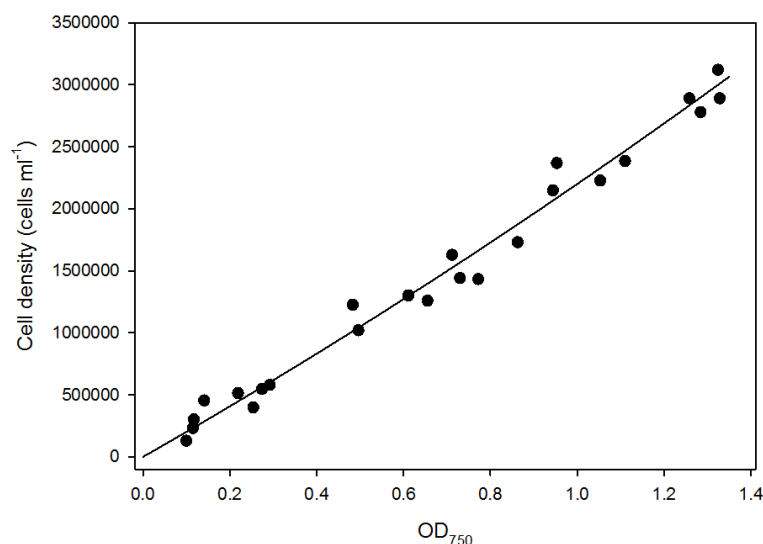


Figure 5.1 Relationship between *C. reinhardtii* cell density and optical density at 750 nm. Datapoints represent individual dilutions from 4 separately cultured populations; line represents quadratic function describing relationship.

#### 5.2.4 S-metolachlor dose response

To determine the response of the stock population to S-metolachlor a dose-response assay was performed. 125000 cells were exposed to a range of doses of S-metolachlor between 0 and 1 mg litre<sup>-1</sup>. After seven days under standard culture conditions, OD<sub>750</sub> was measured at various S-metolachlor doses. The experiment was replicated three times across separate seven day periods. The relationship between S-metolachlor dose and OD<sub>750</sub> was described by a 4 parameter log-logistic function (fig. 5.2, see chapter 3.2.6 for dose-response statistical analysis), which did not fit significantly worse than saturated ANOVA ( $P=1$  to 3 s.f.). The minimum inhibitory concentration (MIC) of S-metalochlor was defined as the lowest dose at which there was no observable growth over the 7 day assay. This value was determined at 0.375 mg litre<sup>-1</sup> (Fig. 5.2).

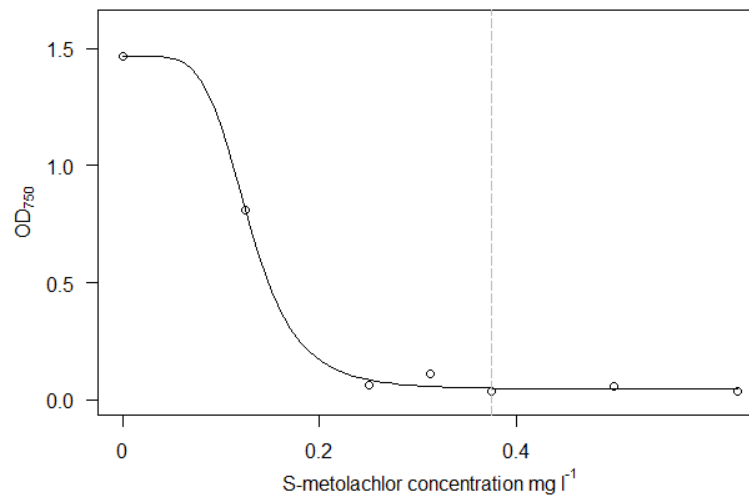


Figure 5.2 S-metolachlor dose-response for *C. reinhardtii* source population after culturing for 7 days. Points are means of three replicate observations, solid line 4-parameter log logistic model. Dashed line represents lowest dose above which there was no observable growth, 0.375 mg litre<sup>-1</sup>, defined as the minimum inhibitory concentration (MIC). Additional 0 responses above 0.6 mg litre<sup>-1</sup> not shown.

### 5.2.5 Herbicide dose cycling regimes

*C. reinhardtii* populations were exposed to three mean dose regimes, so that the mean weekly dose experienced by cultures over the entire experiment was 0.281, 0.375 or 0.469 mg litre<sup>-1</sup> (0.75 MIC, MIC and 1.25 MIC respectively). For each mean dose, independently evolving cultures were exposed to one of four different dosage rotation treatments. At one extreme, cultures were exposed to the same herbicide dose each week (0.75 MIC, MIC or 1.25 MIC). This treatment is subsequently referred to as  $\Delta 0$  rotation. For the other three dose rotation treatments, cultures were exposed to a relatively high and low dose (either side of the mean dose for that regime) over alternating periods. The most extreme dose rotation alternated between exposure to no herbicide and twice the mean weekly dose. This rotation treatment is subsequently referred to as  $\Delta 2$  rotation. The remaining two strategies used dose rotations between these two extremes. One strategy alternated between receiving  $\frac{2}{3}$  and  $\frac{4}{3}$  of the mean dose, a difference between higher and lower rotation doses of  $\frac{2}{3}$ , or 0.66, of the mean dose (subsequently referred to as  $\Delta 0.66$  rotation).

The final strategy alternated between receiving  $\frac{1}{3}$  and  $\frac{5}{3}$  of the mean dose, a difference of  $\frac{4}{3}$ , or 1.33, of the mean dose (referred to as  $\Delta$  1.33 rotation). All doses received are described in table 5.1. For the rotating dose strategies two levels of temporal variation were tested, with dose rotating on a weekly or biweekly basis. All experimental conditions were replicated six times, each replicate originally established from one of the six separate stock populations described above. For selection regimes with rotating doses, three replicates commenced with exposure to the relatively high dose and three with exposure to the relatively low dose. In total there were 126 evolving lines and 6 control populations. The experiment ran for 16 weekly transfers.

Table 5.1 Doses received under all rotation strategies

Difference between doses (proportion of mean dose)	Doses rotated (proportions of mean dose)		Doses received (mg litre <sup>-1</sup> )					
			0.75 MIC		MIC		1.25 MIC	
	<b>High</b>	<b>Low</b>	High	Low	High	Low	High	Low
<b>0</b>	<b>1</b>	<b>1</b>	0.281	0.281	0.375	0.375	0.469	0.469
<b>0.66 x</b>	<b>4/3</b>	<b>2/3</b>	0.187	0.375	0.500	0.250	0.625	0.313
<b>1.33 x</b>	<b>5/3</b>	<b>1/3</b>	0.468	0.094	0.625	0.125	0.782	0.156
<b>2x</b>	<b>2</b>	<b>0</b>	0.563	0	0.750	0	0.938	0

### 5.2.6 Population size measures

At the end of every seven day culture period of the dose rotation regimes, immediately before transfer to the following culture medium, OD<sub>750</sub> was measured and used to estimate cell density according to equation 5.1. Cell density for control lines maintained without herbicide in parallel to selection lines was found to significantly differ between weeks ( $F_{(5,80)} = 3.709$ ,  $P < 0.001$ ). To account for this, cell density for all evolving cultures and standardised assays were converted to proportion of mean control cell density for the week they were cultured. As cell densities represent the extent of population growth during culturing, this measure is subsequently referred to as weekly growth (as a proportion of control).

### 5.2.7 Fitness comparisons of final lines

At the end of the experiment, fitness was compared for all experimental lines at the mean dose to which they had been exposed (0.75, 1 and 1.25 MIC); as well as in the absence of herbicide. Following completion of the 16 week cycling regimes, all populations were transferred to BM and cultured for one week in the absence of herbicide. This additional growth cycle in BM was necessary to multiply all evolved lines in a standard (herbicide-free) environment, thus limiting transfer of herbicide into standard fitness assay conditions and ensure that *C. reinhardtii* cells were free from residual effects of herbicide exposure when growth assays were conducted. To compare growth in their respective mean dose environments, cells were transferred to fresh culture media at the appropriate herbicide dose for that line (0.281, 0.375 or 0.469 mg litre<sup>-1</sup>), and cultured for seven days. To compare growth in the absence of herbicide, cells were transferred to BM and cultured for seven days without herbicide. For all conditions initial population sizes and culture conditions were as described above. For all conditions, at the end of the seven day culture period OD<sub>750</sub> was measured and used to estimate cell density, which was then converted to a proportion of control line growth in the absence of herbicide, as described in section 5.2.4.

### 5.2.8 Statistical analyses

To test the effect of different treatment regimes on the growth of *C. reinhardtii* populations, weekly growth data (section 5.2.4) were converted to mean growth over a four week period (weeks 1-4, 5-8, 9-12 and 13-16). Analysing in this way made direct comparisons possible between dose rotation strategies out of phase with each other (those starting with the relatively higher or lower dose in a cycle) and between weekly and biweekly rotations. Data were analysed in a 4-way repeated measures analysis of variance (ANOVA), with four weekly mean growth data as the dependent variable. The independent variables were: time (4 week period; repeated measures for each independently evolving line at each time point), mean dose, dose rotation strategy, and cycling phase length. The cycling phase length factor was partially nested, as comparisons were only possible where dose rotation strategy was not 0. Where factors or interactions were found to be significant, post-hoc *T*-tests with step-wise Holm-Bonferroni adjustment for multiple comparisons (Holm, 1979) were performed on groups of interest in order to identify which individual treatments were significantly different.

To compare the fitness of evolved lines in the presence and absence of S-metolachlor (section 5.2.5), population size after 7 days (relative to control lines in BM) was used as the dependent variable in 3-way ANOVA tests. The independent variables were mean dose received, dose rotation strategy, and cycling phase length. Post-hoc tests to identify differences between individual treatments were performed as above. All analyses were performed in R (R Development Core Team, 2013).

## 5.3 Results

### 5.3.1 *Chlamydomonas reinhardtii* population dynamics within herbicide rotation strategies

Analysis of variance was performed to test the impact of all experimental factors on mean *C. reinhardtii* population size in order to determine whether dose rotation could reduce *C. reinhardtii* growth compared to receiving a uniform dose. Mean *C. reinhardtii* population size across consecutive four week periods was analysed as a repeated measure (four separate four week periods within the entire experiment) for each independently evolving line, so the analysis of variance tests the effects of the

main experimental factors (mean dose received, dose rotation strategy and rotation phase duration), and how they change over time.

The analysis of variance demonstrated that there was a significant main effect of rotation strategy on *C. reinhardtii* growth over the course of the experiment ( $F_{(3,105)}=7.596$ ,  $P<0.001$ ). Post-hoc comparisons confirmed that mean population sizes in populations exposed to  $\Delta 2$  rotation were greater than the  $\Delta 0.66$  and  $\Delta 1.33$  rotations ( $P<0.01$  for both comparisons), but were not significantly different to  $\Delta 0$  rotation. There were no significant differences between  $\Delta 0$ ,  $\Delta 0.66$  and  $\Delta 1.33$  rotations (fig. 5.3 a).

There was also a significant main effect of mean dose received on *C. reinhardtii* growth ( $F_{(2,105)}=8.461$ ,  $P<0.001$ ). Lines receiving a mean dose of 1.25 MIC showed significantly reduced growth compared to lines receiving a mean dose of 0.75 MIC ( $P<0.01$ ), but neither group showed a significant difference compared to lines under MIC (fig. 5.3 b). There was no overall interaction between the main effects of mean dose regime and rotation strategy, indicating that the effects of dose rotation were consistent across different mean dose regimes, when compared across the entire 16-week experimental period ( $F_{(6,105)}=0.144$ ,  $P=0.99$ ).

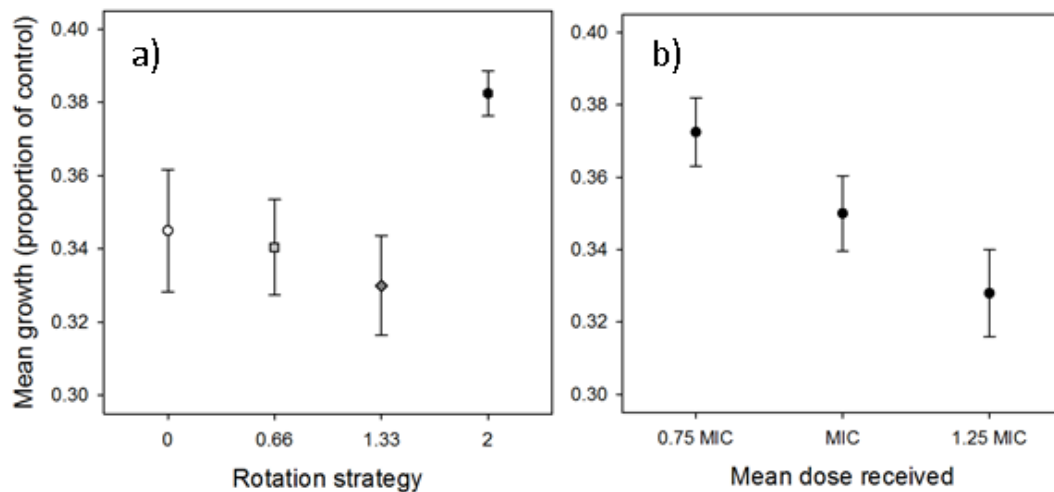


Figure 5.3 Mean *C. reinhardtii* growth (as a proportion of growth of control lines in the absence of herbicide) across entire 16 week experiment for a) four different S-metolachlor dose rotation strategies showing under b) three different mean dose regimes. Datapoints are mean values, bars represent standard error.

However, there were also significant changes in *C. reinhardtii* growth over time (fig. 5.4,  $F_{(3,315)}=681$ ,  $P<0.001$ ). Each consecutive four week period represented a significant increase in mean growth from the previous four week period ( $P<0.01$  for all comparisons). As population size was standardised (at 125000 cells) at the commencement of each weekly transfer, increases in population growth over time (fig. 5.4) indicate adaptation to the herbicide selection environment.

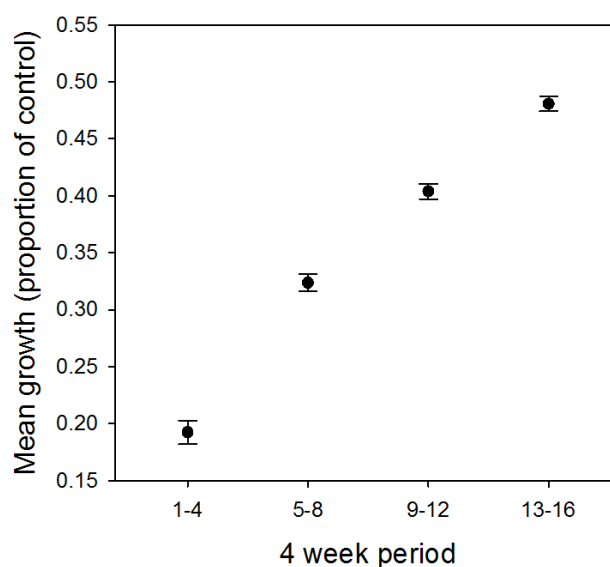


Figure 5.4 Changes in mean *C. reinhardtii* growth (as a proportion of growth of control lines in the absence of herbicide) over time, across four different S-metolachlor dose rotation strategies under three different mean dose regimes. Datapoints are mean values, bars represent standard error.

There was a significant interaction between time, mean dose and rotation strategy on *C. reinhardtii* growth (fig. 5.5,  $F_{(18,315)}=2.717$ ,  $P<0.001$ ). In order to describe these changes over time the  $\Delta 0$  rotation strategy is considered as representing a ‘default’ herbicide application procedure for each mean dose regime, and other rotations are described in relation to it.

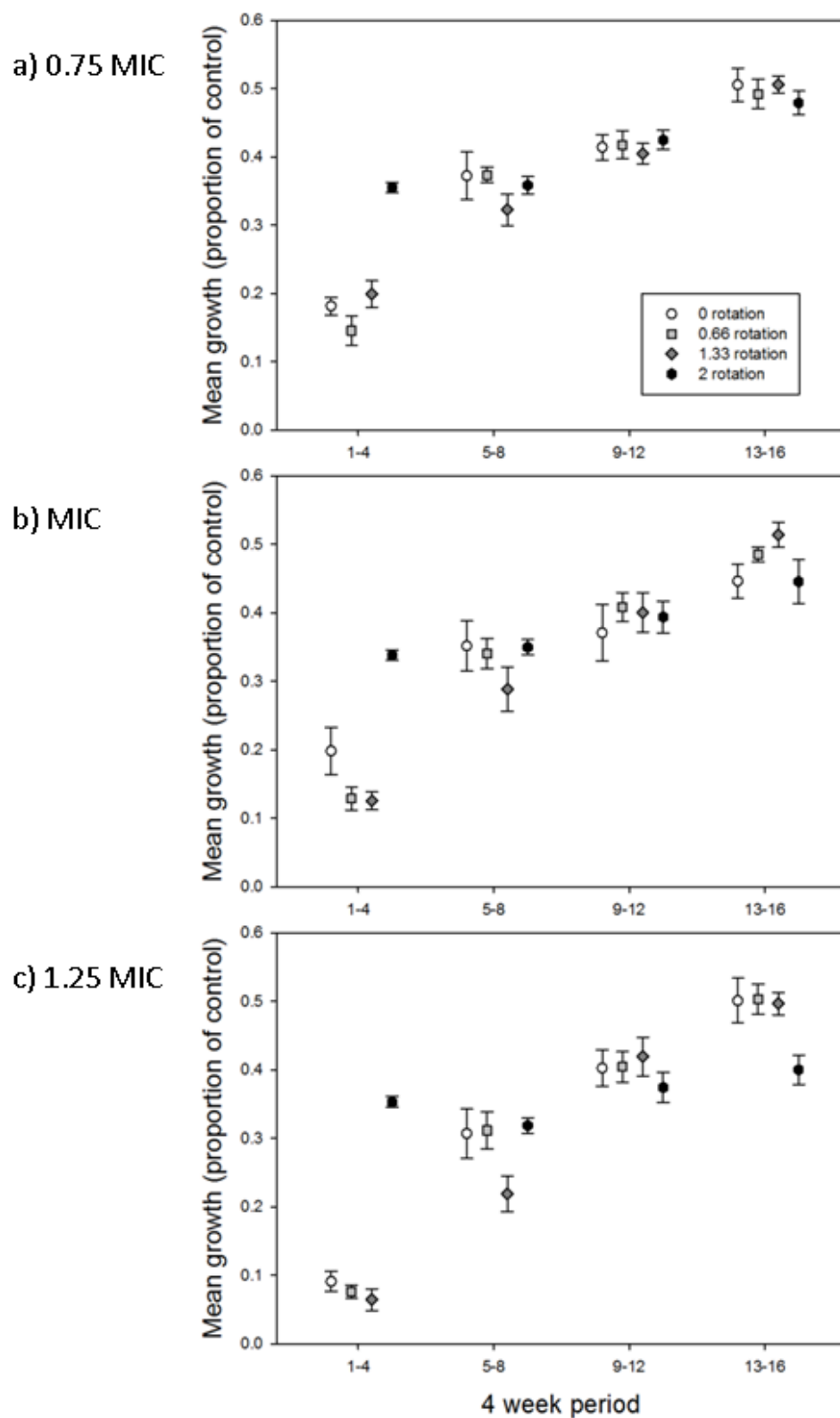


Figure 5.5 Mean *C. reinhardtii* growth (as a proportion of control growth in the absence of herbicide) showing change over time for different herbicide dose rotation strategies, under three different mean dose regimes: a) 0.75 MIC, b) MIC and c) 1.25 MIC. Datapoints are mean values from six replicate lines, bars represent standard error.



During weeks 1-4, mean population sizes were significantly greater for the  $\Delta 2$  rotation strategy at all mean dose doses regimes ( $P < 0.001$  for all pairwise comparisons). No other dose rotation strategies significantly differed from  $\Delta 0$  rotation in the first 4 week period, although there was a strong trend for lower mean growth for the  $\Delta 0.66$  and  $\Delta 1.33$  rotations than  $\Delta 0$  rotation under mean dose MIC (fig. 5.5a,  $P < 0.06$  in both comparisons).

During weeks 5-8, there were no significant differences in mean population size for all rotation strategy and mean dose combinations. However, there was a consistent trend for  $\Delta 1.33$  rotation to result in lower mean *C. reinhardtii* population sizes than every other rotation strategy (including  $\Delta 0$ ) at all mean doses, and the difference between  $\Delta 1.33$  rotation and  $\Delta 0$  rotation was close to significance at the mean dose of 1.25 MIC ( $P < 0.06$ ).

During weeks 9-12, there were no significant differences in mean population size between rotation strategies at all mean doses. During weeks 13-16, mean population size for the  $\Delta 2$  rotation strategy at 1.25 MIC was lower than all other rotation strategies ( $P < 0.05$ ), but there were no significant differences between strategies under the other mean dose regimes.

In general, these results indicate that although  $\Delta 2$  rotation initially resulted in high *C. reinhardtii* population sizes, there was limited increase in growth across time for this rotation strategy, and under the 1.25 MIC mean dose regime this resulted in significantly less growth than other rotation strategies by the end of the experiment. The other most notable trend was for  $\Delta 1.33$  rotation to consistently (though non-significantly) reduce population size compared to all other rotations, including  $\Delta 0$  rotation, within the second four week period. This lack of significance must be considered in light of the strong multiple test adjustments to significance levels when performing post-hoc pairwise tests in a complex multi-factorial experiment. Bonferroni adjustments may be overly conservative, especially if subgroups of tests provide independent information in their own right (Perneger, 1998), as could be argued for comparisons made here within the same mean dose regimes. Relaxing the multiple test adjustment to significance levels suggests that in the first four week period, under the MIC dose regime both  $\Delta 0.66$  and  $\Delta 1.33$  rotations provide superior *C. reinhardtii* control than  $\Delta 0$  rotation ( $P = 0.01$  and  $P < 0.01$ , respectively), and in the

second four week period  $\Delta 1.33$  rotation reduced growth more than  $\Delta 0$  rotation under 1.25 MIC ( $P=0.03$ ).

There was a small but significant overall difference between weekly or bi-weekly rotation phase ( $F_{(1,90)}=7.081$ ,  $P<0.01$ ) with weekly rotation resulting in lower growth than bi-weekly rotation (fig. 5.6). There were no significant interactions between cycling phase duration and any other factors.

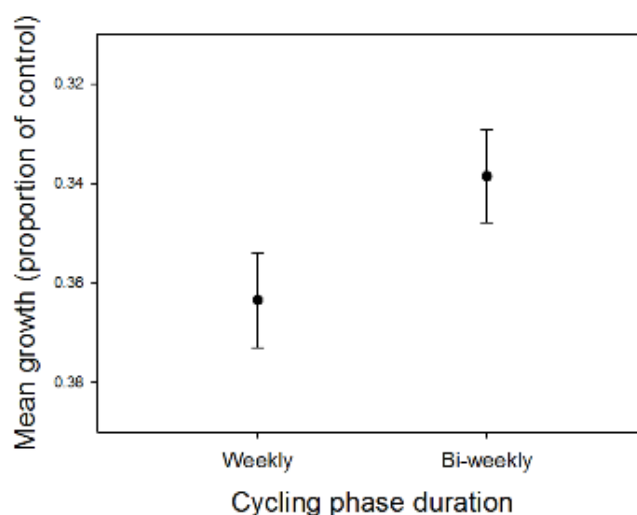


Figure 5.6 Mean *C. reinhardtii* growth (as a proportion of control growth in the absence of herbicide) comparing weekly and biweekly phase duration for four different S-metolachlor dose rotation strategies under three different mean dose regimes across entire 16 week experiment. Datapoints are mean values from six replicate lines, bars represent standard error.

### 5.3.2 *C. reinhardtii* population fluctuations within a single rotation period

As the primary population size analysis (3.1) only considers mean population size within a four week period, any fluctuations in size between the higher and lower doses in a rotation may be overlooked (fig. 5.7). To test how population size dynamics within cycles were affected by mean dose regime and rotation strategy an analysis of variance was performed, comparing the difference in maximum and minimum *C. reinhardtii* population size (subsequently referred to as population fluctuation) within four week periods. There was a significant effect of rotation strategy on population fluctuation ( $F_{(3,105)}=386$ ,  $P<0.001$ ), with increasing difference between doses resulting in greater population fluctuations ( $P\leq 0.01$  for all pairwise

comparisons between rotation strategies). Further, more complex interactions were also present associated with exact difference between doses and population changes over time (data not shown). The details of population fluctuation, and their potential importance, will depend on the biology of the species being controlled, and the management needs in consistency of control, but the effect of rotation increasing fluctuation is highlighted here to indicate considerations which must be made when employing a dose rotation strategy in agricultural scenarios.

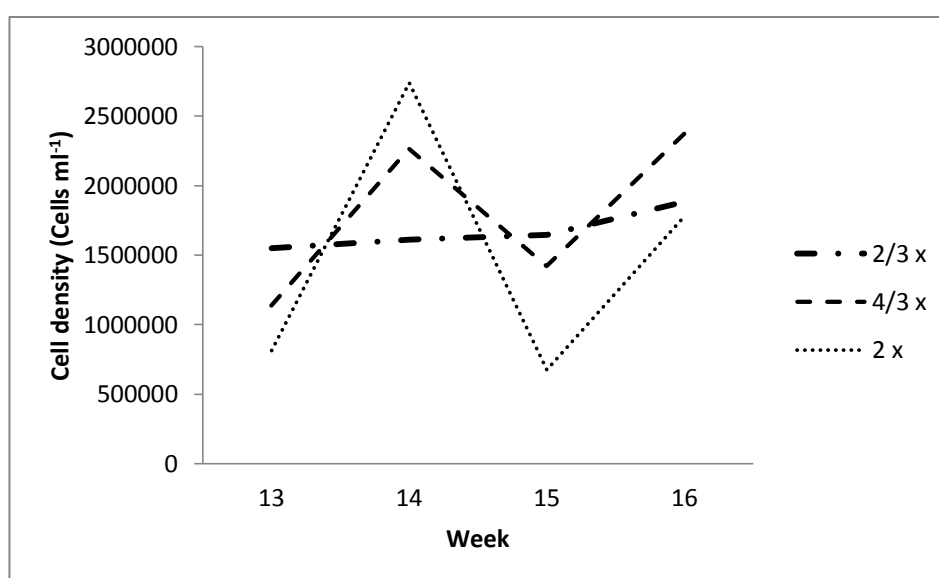


Figure 5.7 Weekly *C. reinhardtii* growth data for single-weekly rotating dose strategies under a mean dose of MIC from week 13 to week 16. Value at each week from 3 replicates (other three replicates not shown, doses received in alternate weeks).

### 5.3.3 Fitness comparisons of final lines in mean dose environment

To compare the level of resistance evolved under each strategy, lines were grown in their respective mean dose environment. Every rotation strategy for all mean doses grew significantly better in their mean dose environment than controls maintained with no herbicide exposure ( $P < 0.001$  in all cases), demonstrating clear adaptation to herbicide in all experimental lines. Analysis of variance was performed testing the effect of mean dose regime, rotation strategy and rotation phase duration on growth in the mean dose environment. There was a significant interaction between rotation strategy and mean dose received (fig. 5.8,  $F_{(6,105)} = 5.507$ ,  $P < 0.001$ ). This effect arose

as under a mean dose of 1.25 MIC, the  $\Delta 2$  rotation strategy showed significantly less growth than the other rotation strategies ( $P < 0.05$  for all comparisons). There were no other significant differences between rotation strategies or mean dose regimes. Cycling phase duration did not show any significant effects or interactions on growth in the mean dose environment.

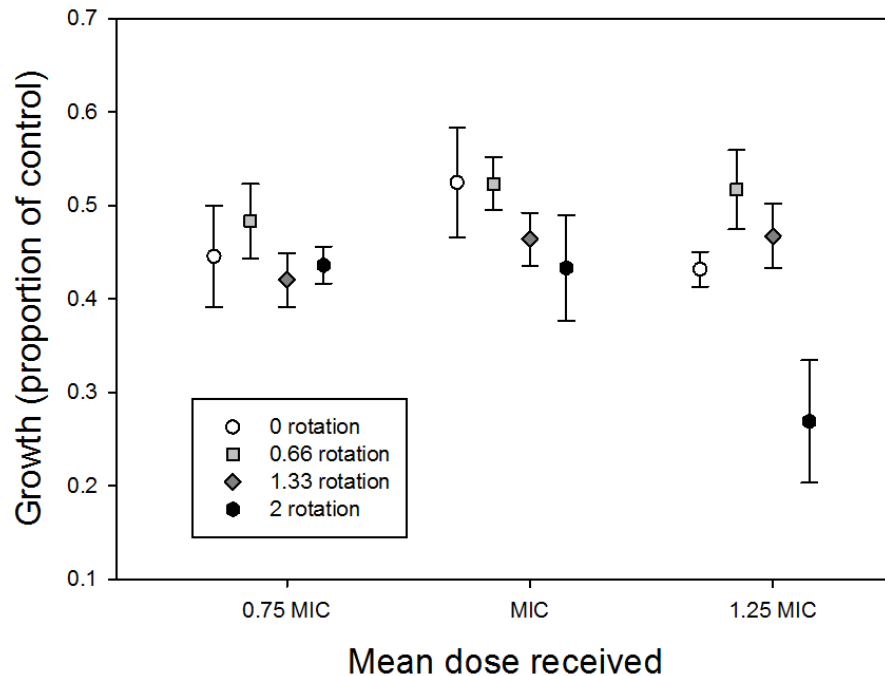


Figure 5.8 Growth of final *C. reinhardtii* populations in the presence of the mean herbicide dose received during selection for lines exposed to four different dose rotation strategies under three different mean dose regimes. Datapoints are mean values, bars represent standard error.

#### 5.3.4 Fitness of *C. reinhardtii* experimental lines in the absence of herbicide

To identify whether different selection regimes had resulted in different fitness costs in the absence of herbicide, analysis of variance was performed testing the effects of mean dose regime, rotation strategy and rotation phase duration on growth of final lines in the absence of herbicide (fig. 5.9). Every experimental line grew significantly less well than controls ( $P < 0.01$  for all comparisons), indicating every selection line showed a fitness cost in the absence of herbicide. There was a

significant effect of mean dose received on growth in the absence of herbicide ( $F_{(2,105)}=11.917$ ,  $P<0.001$ ), with lines from 0.75 MIC regimes showing significantly less growth than lines which had received mean doses of 1.25 MIC ( $P<0.001$ ); but neither showed a significant difference to overall lines grown under MIC. There was also a significant interaction between mean dose received and rotation strategy ( $F_{(6,105)}=3.486$ ,  $P<0.01$ ), but not for rotation strategy alone. This effect arises as for a mean dose of MIC  $\Delta 2$  rotation showed significantly less growth than the other rotation strategies ( $P<0.01$  for all pairwise comparisons), but individual rotation strategies did not significantly differ for other mean doses. Every experimental line grew significantly less well than controls; i.e. every selection line showed a fitness cost in the absence of herbicide ( $P<0.01$  for all comparisons).

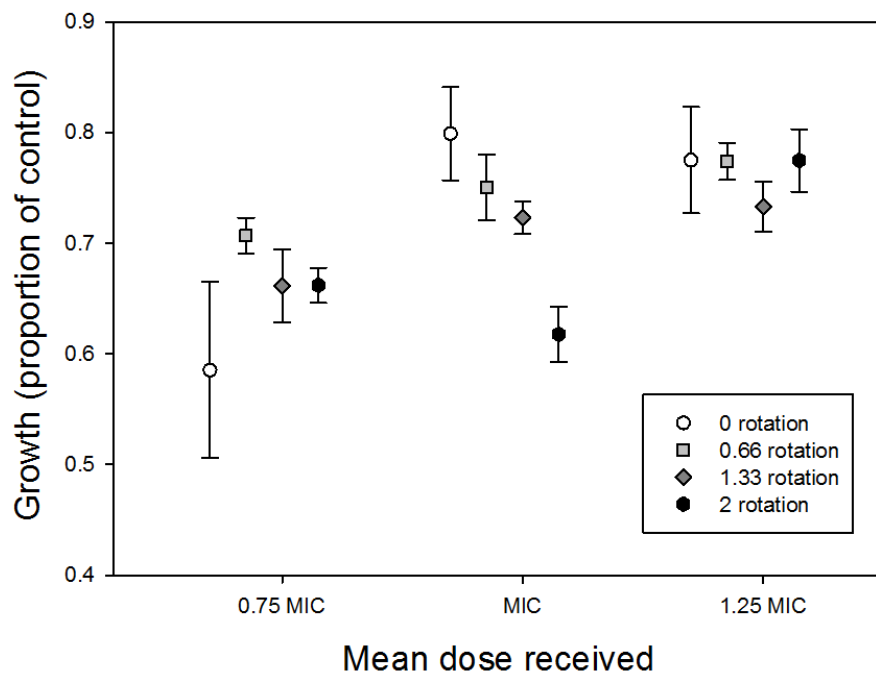


Figure 5.9 Growth of final *C. reinhardtii* populations lines in the absence of herbicide for lines exposed to four different dose rotation strategies under three different mean dose regimes. Datapoints are mean values, bars represent standard error.

## 5.4 Discussion

### 5.4.1 Impact of dose rotation strategies on *C. reinhardtii* growth

This study represents the first empirical test of a herbicide dose rotation strategy. Across the entire experimental period, dose rotation strategies did not reduce *C. reinhardtii* growth compared to uniform dosage. However, dose-rotation strategies also resulted in specific *C. reinhardtii* population size dynamics with potential applications for specific management strategies. Despite the  $\Delta 2$  rotation strategy offering poor overall control, this strategy showed little increase in growth over the course of the experiment, and under the highest mean dose tested (1.25 MIC) eventually provided superior control. This suggests a trade-off in the most extreme dose rotation strategies between successfully limiting, or even preventing, resistance, and large population sizes in the low dose phase, as previously demonstrated in theoretical models (Gardner et al., 1998). There were also strong indications that in the earlier stages of the experiment the intermediate rotation strategies ( $\Delta 0.66$  and  $\Delta 1.33$  rotation) could potentially provide superior control than application of a uniform herbicide dose. Even though these advantages were only observed in the first 8 weeks of the experiment, this period probably represents at least 50 generations of *C. reinhardtii* (Reboud et al., 2007), potentially equivalent to several decades for a typical weedy plant.

The use of low herbicide doses as part of a resistance management strategy is controversial, and several risks have been suggested (Gressel, 2009). Low herbicide doses at which some individuals can survive may create a highly stressful environment, increasing mutation rate, and potentially supplying resistance mutations (Gressel, 2011). More broadly, where one environment is easier to adapt to (i.e. under lower doses), even small increases in fitness can result in greater population sizes and subsequently enhanced mutation supply for further adaptation (Campos and Wahl, 2010). Mutations selected for in response to one environment may also alter the mutational landscape of genome such that further mutations, of potential benefit in an alternative environment, are more likely (Yeh et al., 2009). Despite these concerns, there were no indications that the rate of resistance evolution increased under dose rotation, over 100 generations of herbicide exposure. However, caution must be taken in applying these dynamics to higher plant systems, as the

NTSR mechanisms thought to be selected for by lower herbicide doses may also provide resistance to alternative herbicide modes of action (Powles and Yu, 2010), and so any strategy with the potential to favour NTSR might also result in cross-resistance to multiple herbicides

#### **5.4.2 Herbicide resistance evolution under dose rotation strategies**

By the end of the experiment, almost all experimental lines displayed similar levels of resistance when grown in the mean dose to which they had been exposed, suggesting dose rotation selected for similar fitness in the intermediate dose environment. The only exception to this was at the most extreme rotation strategy tested,  $\Delta 2$  rotation under a mean dose of 1.25 MIC. If environments in a rotation are too different, it may not be possible for adaptation to both environments, and a generalist phenotype will not evolve (Kassen, 2002). This may result in evolution of repeated, alternating specialisations to the individual environments, as has been demonstrated for bacteriophage adaptation to alternating hosts (Crill et al., 2000). Very extreme dose rotation strategies may present such opportunities in order to maintain long term population control.

More frequent rotation may be expected to result in a more heterogeneous environment, more likely to select for generalists (Collins, 2011). However, in this study the dynamics under both cycling frequencies were the same, and both show similar fitness in the mean dose, at the end of the experiment. The increased cycling frequency appears to have selected for increased niche width, so there was increased fitness in the two alternating doses, and hence overall increased growth (Kassen, 2002), but no impact on fitness in the intermediate mean dose. Temporal environmental heterogeneity has been shown to give rise to a number of complex and often transient effects, including coexistence of multiple evolutionary strategies within a single population (Venail et al., 2011). Further experimentation would be required to fully interpret these cycling frequency dynamics at different stages in the experiment and determine where effects are due to a universal resistance phenotype, or a result of a single population containing multiple evolutionary paths.

Similarly to the effect of growth in the mean dose environment, in most cases rotation strategy did not impact on the strength of fitness trade-offs in the absence of herbicide, with every line displaying a significant fitness trade-off. Lines selected in

lower mean dose regimes showed greater fitness trade-offs in the absence of herbicides. Previous studies have compared *C. reinhardtii* fitness in different herbicide doses in a local adaptation context (Lagator, 2012), to test the effect of environmental difference on fitness (Kawecki and Ebert, 2004). In contrast to this study, growth in the absence of herbicide did not differ between lines selected at different S-metolachlor doses (Lagator, 2012). As above, further study would be required to determine how dose rotation has changed this dynamic, and whether the effects are a result of individual evolutionary strategies or a population level response.

### **5.4.3 Considerations for resistance management in agriculture**

The results in this study suggest some potential to employ dose rotation strategies to limit herbicide resistance, and do not indicate that these strategies pose any risks compared to uniform dosage. However, the experiment must be considered in light of the differences between *C. reinhardtii* and higher plant weeds. The difference in generation time between *C. reinhardtii* and weedy plants may have an impact on dynamics observed here. As each rotation phase in this experiment occurred over several generations of *C. reinhardtii*, the two levels of temporal variation tested were both very coarse grained. In weedy plants a more fine-grained level of temporal variation can be employed. Rotating every single generation, instead of across multiple generations, could result in changes to dynamics and additional effects not observed here. Strategies may even be considered that cycle multiple herbicides within the lifetime of an individual plant. Whether environmental heterogeneity is encountered within the life of a single individual is expected to have significant impacts on the results of selection (Kassen, 2002). If a single individual is exposed to multiple environments, it might be expected to evolve a broad generalist strategy to cope with them all; however, if the environment changes at a slower rate than individual lifespan, a generalist phenotypic plasticity might be expected, where individuals respond developmentally by changing phenotype according to their environment (Levins, 1968). Both forms of environmental heterogeneity are encountered by weedy plants in typical herbicide control. There is exposure to both pre- and post-emergence herbicides at different stages in an individual's lifecycle, but then the dose or mode of action of either (or both) may be varied between generations.



Further difficulties may be encountered in using dose rotation strategies in agricultural systems. As suggested in models (Gardner et al., 1998) and supported by some lines in this experiment, the success of dose rotation strategies depends upon the relatively high dose being sufficiently strong. The environmental impacts of high herbicide doses must be considered in management strategies though (Blackshaw et al., 2006; Pimentel, 2005), and it may be that even if overall herbicide level is the same as a non-rotation strategy, the high dose is too environmentally damaging to be used in practice. The greater population variability under dose-rotation strategies may be problematic in agricultural systems. Even if mean control is high, low dose years may result in weed population sizes so great that the strategy is uneconomic overall. However, if the herbicide rotation strategy is tied into field management rotations that can cope with the impact of weeds in low or 0 dose periods, for example in different crop rotations or fallow years, then high rotations may still prove useful.

Other important differences in weedy plant management may arise from population dynamic effects not included here. In this system initial population sizes were standardised at the beginning of every rotation, with susceptible immigrants contributing if there was low survival in the previous rotation. In an agricultural system similar effects may arise if seeds remain viable in the soil for several years, and there is high population growth in low dose periods. However, if the low dose rotation provides a degree of control which reduces population size in the following generation such that populations are below carrying capacity there may be a gradual reduction in population size not accounted for in the experiment. These reduced population sizes may provide an additional effect of decreasing the incidence of resistance conferring mutations, slowing the evolution of resistance.

Finally, *C. reinhardtii* is a haploid and asexual organism (under the experimental conditions of this study), and as such cannot capture some higher plant dynamics. In a diploid system the mode of inheritance and the heritability of resistance can have major impacts on its evolution (Diggle and Neve, 2001; Gardner et al., 1998). The range of different breeding systems possible in higher plants can also determine the evolution of resistance (Jasieniuk et al., 1996). Sexual reproduction may also make a number of spatial effects important in field scenarios. Within fields non-random mating could concentrate high levels of resistance among individuals in the same

area, and between fields there may be gene flow between independently evolving metapopulations.

## 6. General Discussion

### 6.1 Research overview

Herbicide resistant weeds represent a major problem for contemporary agriculture (Zimmer, 2013). Reported cases of herbicide resistance continue to increase (Heap, 2014a), but maintaining weed control is essential for sufficient yields (Oerke, 2006). Technological advances have failed to provide weed management solutions, with concerns raised over the potential for herbicide resistant GM crops to exacerbate resistance evolution in weeds (Nature editorial, 2014), and a lack of new chemical options to replace ineffective herbicides (Duke, 2012). In light of these problems, a change in our approach to herbicide resistance research has been suggested, with a focus on the ecological and evolutionary dynamics that lead to herbicide resistance potentially highlighting opportunities for long term, sustainable weed management (Neve et al., 2009). This study explored the evolution of herbicide resistance in *Alopecurus myosuroides* (black-grass) in this evolutionary ecology context, supplemented with a test of evolutionarily-aware management using the model organism *Chlamydomonas reinhardtii*.

#### 6.1.1 Variation in herbicide response in susceptible *A. myosuroides*

Characterisation of herbicide resistant weeds frequently focuses on major effect target-site resistance genes which provide a high level of resistance (Jasieniuk et al., 1996). Rare mutations resulting in major effect target-site resistance will be rapidly selected for, and spread throughout a field population (Renton et al., 2011). However, it is now recognised that resistance also occurs as a polygenic trait, and pre-existing diversity in this polygenic resistance may confer high levels of standing variation in herbicide sensitivity, even in previously unexposed populations (Neve and Powles, 2005a; Busi et al., 2012). In chapter 2, this variation in response to low herbicide doses was tested for *A. myosuroides* populations with no prior history of herbicide application. Variations in response were observed following application of two commonly used herbicide modes of action: the ACCase inhibitor fenoxaprop-P-ethyl and the ALS inhibitors mesosulfuron-methyl and iodosulfuron-methyl-sodium. An additional ACCase inhibiting herbicide, cycloxydim, did not exhibit this variation in herbicide response, suggesting that the pre-existing polygenic resistance

mechanisms in the *A. myosuroides* populations tested are specific to individual herbicide chemistries.

### **6.1.2 Selection for herbicide resistance using reduced herbicide rates**

If additive genetic variation is responsible for the phenotypic variability in herbicide response demonstrated in chapter 2, polygenic resistance would be expected to show quantitative inheritance, with selection rapidly increasing resistance (Busi et al., 2012; Neve and Powles, 2005b). Quantitative inheritance of resistance has important implications for prediction and control of resistance evolution (Neve et al., 2014), and informs ongoing debate over appropriate herbicide dose rates to balance the risks of resistance with environmental and economic concerns (Gressel, 2009). In chapter 3, the potential for selection within the range of pre-existing variation in herbicide response to increase resistance was demonstrated in susceptible *A. myosuroides*, using the ACCase inhibiting herbicide fenoxaprop-P-ethyl. The results demonstrated that selection using low herbicide rates can rapidly and significantly increase resistance, but that the dynamics depend on dose and population size. At the lowest population sizes exposed to herbicide (100 individuals) there were non-significant increases in resistance after a single generation of selection (inferred by LD<sub>50</sub> following dose-response assays). For the two larger population sizes (200 and 600 individuals), selection at both doses tested (13.6 and 27.2 g fenoxaprop-P-ethyl ha<sup>-1</sup>) significantly increased resistance. A second round of selection was performed on the progeny of one of these selected groups (27.2 g fenoxaprop-P-ethyl ha<sup>-1</sup> from 200 plants), testing selection using the same dose, and an increased dose (48 g fenoxaprop-P-ethyl ha<sup>-1</sup>), which resulted in further, but non-significant, increases in resistance. Finally, relaxation of selection was tested, allowing the same selected progeny group to cross without any herbicide application. Progeny following relaxation of selection showed some return to susceptibility in mortality response, with an LD<sub>50</sub> between that of the original population and the selected group.

### **6.1.3 Fitness and competitive impacts of selection for herbicide resistance**

Herbicide resistance might be expected to confer a fitness cost in the absence of herbicide (Purrington and Bergelson, 1996). The presence of any fitness costs could

influence the evolution and spread of resistance, and may be exploited in management strategies that maintain weed control while limiting or delaying resistance evolution (Vila-Aiub et al., 2011). In chapter 4, a competition experiment was reported that tested whether selection for resistance in *A. myosuroides* (in the progeny following two generations of selection at 27.2 g fenoxaprop-P-ethyl ha<sup>-1</sup> from 200 individuals), had any impact on fitness and competitive ability compared to the original susceptible population. Broadly, there were no significant differences in plant fitness or competitiveness between the resistant and susceptible populations when competing with each other, except at the very highest sowing density tested, where the resistant population was significantly less competitive. There were no significant differences in fitness or competitiveness between the groups when growing with wheat, where both resistant and susceptible populations were highly competitive. This competitiveness against the crop highlights the importance of *A. myosuroides* control (Moss, 2013b), but the similarity in fitness and competitiveness between resistant and susceptible populations suggest that at this stage in resistance evolution, resistant plants are likely to be problematic, with no fitness trade-offs to exploit in management and control.

#### **6.1.4 Dose rotation strategies tested in *Chlamydomonas reinhardtii***

Different herbicide doses may select for different resistance mechanisms, and so rotating between doses might reduce continuous selection for any one resistance mechanism and ultimately limit resistance evolution (Gardner et al., 1998). Studies testing herbicide management strategies are difficult to perform in weed species, due to the generation time and growing space required (Reboud et al., 2007). Model organisms can overcome some of these constraints (Buckling et al., 2009), testing principles which may transfer to higher plant systems. In chapter 5, *Chlamydomonas reinhardtii* was used to compare dose rotation strategies with constant application of a single herbicide dose. Rotating between no herbicide application and a very high dose successfully limited resistance evolution, but with large mean population sizes. There was a consistent but non-significant trend for intermediate dose rotations to limit the evolution of resistance during the early stages of the experiment, but across the whole experimental period these intermediate rotations had the same effect as applying a single herbicide dose.

## 6.2 Research in context

Rapid selection for resistance by applying a relatively low herbicide dose (within the range of standing genetic variation for herbicide susceptibility) has previously been shown in *Lolium rigidum* (e.g. Neve and Powles, 2005a; Busi and Powles, 2009), and this study demonstrates the same dynamic occurring in *Alopecurus myosuroides*. An appreciation of this ‘creeping resistance’ is essential to understand and prevent herbicide resistance problems escalating (Neve et al., 2014), and so the principles demonstrated here must be factored in to future *A. myosuroides* management. The study suggests there is considerable pre-existing variation in herbicide response in susceptible *A. myosuroides* populations, which can recombine and rapidly increase resistance, and this may in part be responsible for its propensity to evolve resistance. Our understanding of the eco-evolutionary dynamics of selection for herbicide resistance can explain why some plants appear to evolve resistance much more readily than others (Heap, 2014b), and anticipate where future problems might arise.

The principles demonstrated experimentally here, in both *A. myosuroides* and *C. reinhardtii* systems, could now be used in models to predict resistance and develop management strategies for agricultural systems (Renton et al., 2014). Theoretical models can use the changes in resistance profile observed following selection to test the hypothesised mechanistic basis for quantitative resistance (Manalil et al., 2012a). Simulation models can use the phenotypic and genetic information gained through experimentation to explore the potential consequences of resistance management strategies (Neve et al., 2011). Experiments describing the fitness and competitive ability of *A. myosuroides*, or other weeds, can then refine these models to accurately simulate growth in field conditions (Colbach et al., 2007) and incorporate fitness trade-offs (Roux et al., 2008).

There must then be effective knowledge transfer to ensure that best practice for weed management is communicated to the agrochemical industry, regulatory bodies, and the end-user (Beckie, 2011). Despite recommendations for resistance management, contemporary farming frequently fails to diversify herbicide control methods (Prince et al., 2012) and/or introduce cultural controls (Riar et al., 2013). In turn, research must also respond to applied issues as they are encountered in real farming systems

(Shaw et al., 2009) and make the case for management recommendations within the economic constraints facing farmers (Riar et al., 2013).

## **6.3 Suggestions for further research**

### **6.3.1 Further selection experiments**

Several additional hypotheses could be explored through further selection experiments. Repeating the experiment performed in chapter 3 with additional population sizes would provide insight into the pre-existing genetic variation in herbicide response, and how sensitive selection is to the extent of variation captured within population subsamples. In chapter 2, the uniform response observed following cycloxydim application was suggested as indicating a lack of genetic diversity from which increased resistance could be selected. Selection experiments similar to those performed in chapter 3, but instead selecting using cycloxydim, could test this theory. Expanding this principle further, other herbicides with alternative modes of action, such as the ALS inhibitors mesosulfuron-methyl and iodosulfuron-methyl-sodium used in chapter 2, could also be used to compare responses to low dose selection. Similar selection in *L. rigidum* has shown selection with one herbicide can result in resistance to herbicides with independent modes of action (Neve and Powles, 2005b; Busi and Powles, 2013). Following selection using fenoxaprop-P-ethyl, or any other herbicides, further characterisation of the progeny could also be performed to test for cross-resistance developing in the same way in *A. myosuroides*.

Due to the time and space requirements, only two successive generations of selection were tested in chapter 3. Though non-significant, increases in resistance profile were still observed following the second generation of selection, and experiments using *L. rigidum* indicate that selecting within pre-existing genetic diversity can result in continued, dramatic increases in resistance for several generations (Neve and Powles, 2005b). Continued selection might seek to establish the maximum extent to which pre-existing genetic variation can increase resistance. Given more time, selection experiments might be used to test potential management strategies, including the dose rotation strategy examined in chapter 5, or commonly

recommended practices such as cycling herbicide modes of action or applying mixtures of herbicides (Beckie and Reboud, 2009).

Alternatively, a divergent recurrent selection approach could be used, to select for increased herbicide susceptibility in parallel with selection for resistance. This technique has been demonstrated in *Lolium rigidum*, exposing individual plant clones to low herbicide doses in order to select the most susceptible plants (Manalil et al., 2012b). Selection for increased susceptibility could provide further insight into the level of pre-existing variation in minor effect resistance genes. Selection for increased susceptibility could also be used to generate plants for further fitness experiments, maximising the difference between resistant and susceptible populations.

### **6.3.2 Resistance mechanisms**

This study focussed on the phenotypic outcomes of selection, but the populations resulting from selection in chapter 3 could provide valuable insight into the underlying molecular mechanisms conferring resistance and their genetic architecture. Modern ‘omics’ (genomics, metabolomics, proteomics and transcriptomics) are now being used to identify candidate genes contributing to a polygenic trait such as NTSR, and begin to establish their function (Gaines et al., 2014). The inheritance of resistance mechanisms can be used to design models which predict the spread of resistance and can test management strategies, as described above (Renton et al., 2014), and an understanding of the molecular basis for quantitative resistance may present new opportunities for weed management (Shaner and Beckie, 2014).

### **6.3.3 Population Screening**

As discussed in chapter 2, the initial stages of selection for herbicide resistance will depend upon the occurrence of major effect TSR mutations and the extent of standing genetic variation for NTSR mechanisms. To establish the frequency of mutations and the prevalence of TSR in field populations known mutations, including mutations for ALS and ACCase inhibitor resistance in *A. myosuroides* (Délye and Boucansaud, 2008; Délye et al., 2011b), can be detected using PCR-based assays (Burgos et al., 2013). As other resistance mechanisms are characterised,



similar PCR techniques could also be used to detect NTSR alleles. As the ‘-omics’ techniques described above become cheaper and more practical, these may also be routinely employed to screen populations for various resistance mechanisms. Molecular and genetic characterisation can be complemented with techniques to screen large numbers of individuals at the phenotypic level. For example, the Syngenta ‘RISQ’ test grows grass seedlings on agar containing various herbicide concentrations to quantify individual plant sensitivity, allowing large numbers of plants to be assessed without the space or time required to perform a whole-plant experiment (Kaundun et al., 2011). Employed together, these techniques will enable extensive monitoring of the evolution of herbicide resistance, experimentally and in the field, and track the underlying mechanisms responsible.

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